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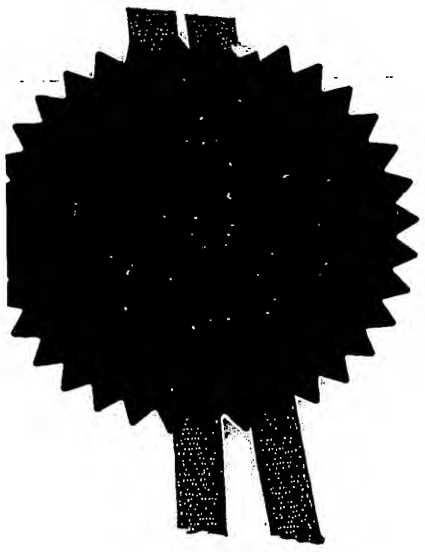

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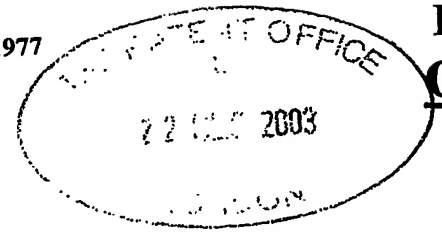

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Glaxo Group Limited
Glaxo Wellcome House, Berkeley Avenue,
Greenford, Middlesex UB6 0NN, Great Britain

Patents ADP number *(if you know it)* 4735870003

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

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G M P Lawrence

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PB60607P

Method

Field of the Invention

This invention relates to methods of modulating the proteolytic processing of amyloid precursor protein (APP) and methods of treatment of conditions in which abnormal processing of amyloid precursor protein (APP) is implicated such as Alzheimer's disease.

Background to the Invention

Alzheimer's disease (AD) is characterised by the presence of two diagnostic features of pathology. These are amyloid plaques and neurofibrillary tangles composed of aggregated beta-amyloid peptide (Aβ40 and Aβ42) and hyperphosphorylated tau respectively (Dawbarn & Allen 2001 Neurobiology of Alzheimer's Disease OUP).

A comprehensive study has shown a strong link in patients between beta-amyloid accumulation and cognitive decline (Naslund et al 2000). This is consistent with genetic and epidemiological studies that suggest that some mutations in APP and presenilin genes can predispose to early onset AD, which mutations also enhance the levels of Aβ40 and Aβ42 peptide, including the ratio thereof.

Cleavage of the type I transmembrane amyloid precursor protein (APP) by two distinct proteases designated beta- and gamma-secretase is necessary for the formation of beta-amyloid peptide. The molecular identity of beta-secretase as the aspartyl-protease Asp2/BACE1 has been confirmed (Hussain et al 1999; Vassar et al 1999). The nature of gamma-secretase remains the source of some debate and is likely to consist of a high molecular weight complex consisting of at least the following proteins: presenilins, Aph1, Pen2 and nicastrin (reviewed in Medina & Dotti Cell Signalling 2003 15(9):829-41).

The processing of APP within the CNS is likely to occur within a number of cell-types including neurons, oligodendrocytes, astrocytes and microglia. While the overall rate of APP processing in these cells will be influenced by the relative level of expression of APP, BACE1/Asp2, presenilin-1 and -2, Aph1, Pen2 and nicastrin.

Furthermore, additional factors regulating the subcellular location of APP can also influence its processing as shown by the finding that mutation of the YENP motif in the APP cytoplasmic domain which blocks its endocytosis reduces beta-amyloid production (Perez et al 1999 J Biol Chem 274 (27) 18851-6). Retention of the APP-beta-CTF in the ER by the addition of the KKQN retention motif is sufficient to reduce amyloid production in transfected cells (Maltese et al 2001 J Biol Chem 276 (23) 20267-20279). Conversely, elevation of endocytosis, by overexpression of Rab5 is sufficient to elevate amyloid secretion from transfected cells (Grbovic et al 2003 J Biol Chem 278 (33) 31261-31268).

Consistent with these findings further studies have shown that reduction of cellular cholesterol levels (a well known risk factor for AD) reduced beta-amyloid formation. This change was dependent on altered endocytosis as demonstrated by the use of the dominant negative dynamin mutants (K44A) and overexpression of the Rab5 GTPase activating protein RN-Tre (Ehehalt et al 2003 J Cell Biol 160 (1) 113-123).

Cholesterol rich microdomains or rafts are also an important cellular site of beta-amyloid production and APP, BACE1 and components of the gamma-secretase complex have all been shown to transiently reside within rafts. Antibody cross-linking of APP and BACE1 towards cholesterol rich rafts was able to elevate beta-amyloid production (Ehehalt et al 2003 J Cell Biol 160 (1) 113-123). Expression of GPI-anchored BACE1, which is exclusively targeted to lipid rafts, is similarly able to elevate APP cleavage and beta-amyloid production (Cordy et al 2003 PNAS 100(20) 11735-11740).

Neuronal growth is finely balanced by the activities of positive factors such as neurotrophins like NGF and matrix proteins like laminin and the opposing activities of inhibitory factors such as ephrins, MAG, semaphorin-3A and Nogo-A (reviewed in Tessier-Lavigne & Goodman 2000 Science 287(5454):813-4). The antagonistic nature of the signalling from these factors is illustrated by the finding that enhanced neuronal growth correlates with a reduction in RhoA activation (Nusser et al 2002 J Biol Chem 277 (39) 35840) and the inhibition of neuronal growth results in increased RhoA activation (Niederost et al 2002 J Neuroscience 22(23) 10368-10376).

NOGO is a protein found in the myelin sheath that has inhibitory action on axonal growth (Prinjha, R et al (2000) Nature 403, 383-384; GrandPre, T et al (2000) Nature 403, 439-444 and Chen, MS et al (2000) Nature 403, 434-439).

At least three Nogo isoforms are generated by alternative splicing of transcripts derived form the NogoA gene. The C-terminal third of all three isoforms shares high homology (approximately 70% at the amino acid level) with the reticulon protein family. NogoA, the largest isoform, has been shown to inhibit axon regeneration in culture. It is thought that the normal role of Nogo proteins is to prevent axon sprouting in the uninjured central nervous system. NogoA is localised to central nervous system myelin and is highly expressed in oligodendrocytes; NogoB and NogoC are expressed in some neurons and several non-neural tissues. All Nogo isoforms surprisingly have a C-terminal ER-retention motif but at least some of NogoA protein is thought to reach the cell surface. All 3 Nogo isoforms have 2 potential trans-membrane domains. Both the C and N termini may be cytoplasmically exposed and a 66 amino acid loop separated by the TM domains may be located extracellularly.

It has been reported that BACE and NOGO may interact in a physical sense, but such reports are based solely on immunoprecipitation experiments (for example WO02/058323 and WO03/088926). There has been no further confirmation of this proposed physical interaction and no experimental evidence of any functional interaction between BACE and NOGO.

The process of neurodegeneration underlies many neurological diseases/disorders including, but not limited to, acute diseases such as stroke, traumatic brain injury and spinal cord injury as well as chronic diseases including Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis and inclusion body myositis.

The identification of therapeutic and prophylactic treatments for neurodegenerative diseases, in particular Alzheimer's disease, remains a highly desirable goal for the pharmaceutical industry and for healthcare providers.

Summary of the Invention

The present invention is based on the finding that antagonists of NOGO influence the production of the amyloidogenic Aβ peptide. This novel finding provides an unexpected route for therapeutic intervention in disorders resulting from abnormal production of Aβ peptide in particular Alzheimer's disease.

Accordingly the invention provides a method of modulating Aβ peptide production using an antagonist of NOGO. Further the invention provides methods of modulating BACE activity, methods of modulating amyloid deposition, pharmaceutical compositions and methods for the treatment of neurodegenerative diseases such as Alzheimer's disease.

Detailed description of the invention

In a preferred aspect the invention provides a method of modulating production of an amyloidogenic peptide comprising contacting a cell which is expressing:

a) the precursor from which the amyloidogenic peptide is derived; and

b) a NOGO polypeptide

with a NOGO antagonist.

In a preferred embodiment the precursor is APP. Preferably the amyloidogenic peptide is Aβ, most preferably Aβ40, Aβ42 or a combination of both.

In a further preferred embodiment the NOGO polypeptide is NOGO-A.

The NOGO antagonist may be a small organic molecule, peptide, polypeptide or antibody that binds to NOGO and thereby inhibits or extinguishes NOGO activity. Other possible NOGO antagonists include peptides, polypeptides, oligonucleotides or polynucleotides (for example aptamers) that binds to the same site on the NOGO receptor molecule as the NOGO polypeptide, but without inducing NOGO receptor-induced activities, thereby preventing the action of the NOGO receptor by excluding NOGO from binding.

Further potential antagonists include small molecules which bind to and occupy a binding site on the NOGO polypeptide thereby preventing binding to cellular binding molecules, such as receptor molecules, such that normal NOGO biological activity is prevented or reduced. Examples of such small molecules include, but are not limited to small organic molecules, peptides or peptide-like

molecules, polypeptides, oligonucleotides or polynucleotides (for example aptamers).

In still another approach, expression of the gene encoding endogenous NOGO polypeptide can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or separately administered (see, for example, O'Connor, J Neurochem (1991) 56:560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). Alternatively, oligonucleotides which form triple helices ("triplexes") with the gene can be supplied (see, for example, Lee *et al.*, Nucleic Acids Res (1979) 6:3073; Cooney *et al.*, Science (1988) 241:456; Dervan *et al.*, Science (1991) 251:1360). These oligomers can be administered *per se* or the relevant oligomers can be expressed *in vivo*. Synthetic antisense or triplex oligonucleotides may comprise modified bases or modified backbones. Examples of the latter include methylphosphonate, phosphorothioate or peptide nucleic acid backbones. Such backbones are incorporated in the antisense or triplex oligonucleotide in order to provide protection from degradation by nucleases and are well known in the art. Antisense and triplex molecules synthesised with these or other modified backbones also form part of the present invention.

In addition, expression of the NOGO polypeptide may be prevented by using ribozymes specific to the NOGO mRNA sequence. Ribozymes are catalytically active RNAs that can be natural or synthetic (see for example Usman, N, et al., Curr. Opin. Struct. Biol (1996) 6(4), 527-33.) Synthetic ribozymes can be designed to specifically cleave NOGO mRNAs at selected positions thereby preventing translation of the NOGO mRNAs into functional polypeptide. Ribozymes may be synthesised with a natural ribose phosphate backbone and natural bases, as normally found in RNA molecules. Alternatively the ribozymes may be synthesised with non-natural backbones to provide protection from ribonuclease degradation, for example, 2'-O-methyl RNA, and may contain modified bases.

Preferably the NOGO antagonist is an antibody, more preferably a monoclonal antibody.

Preferably the monoclonal antibody is a function-blocking anti-NOGO monoclonal antibody, more preferably a function-blocking anti-NOGO-A monoclonal antibody.

Preferably the function-blocking anti-NOGO-A monoclonal antibody comprises CDR's as shown in the tables 1 to 6 which show the CDRs of three independently isolated anti-NOGO antibodies: 2A10/3, 2C4/1 and 15C3/3. The CDR's are identified as described by Kabat (Kabat et al. (1991) "Sequences of proteins of immunological interest"; Fifth Edition; US Department of Health and Human Services; NIH publication No 91-3242). CDRs preferably are as defined by Kabat but following the principles of protein structure and folding as defined by Chothia and Lesk, (Chothia et al., (1989) "Conformations of immunoglobulin hypervariable regions"; Nature 342, p877-883) it will be appreciated that additional residues may also be considered to be part of the antigen binding region.

Table 1: Antibody 2A10/3 light chain CDRs

CDR	*According to Kabat*
L1	RSSKSLLYKDGKTYLN (SEQ ID NO:1)
L2	LMSTRAS (SEQ ID NO:2)
L3	QQLVEYPLT (SEQ ID NO:3)

Table 2: Antibody 2A10/3 heavy chain CDRs

CDR	*According to Kabat*
H1	SYWMH (SEQ ID NO:4)
H2	NINPSNGGTNYNEKFKS (SEQ ID NO:5)
H3	GQGY (SEQ ID NO:6)

Table 3: Antibody 2C4/1 light chain CDRs

CDR	*According to Kabat*

L1	RSSQSLVHSNGNTYLH (SEQ ID NO:7)
L2	KVSNRFS (SEQ ID NO:8)
L3	SQSTHVPLT (SEQ ID NO:9)

Table 4: Antibody 2C4/1 heavy chain CDRs

CDR	***According to Kabat***
H1	FSCYAMS (SEQ ID NO:10)
H2	SISDGGSYTYYPDNVKG (SEQ ID NO:11)
H3	ELLFDY (SEQ ID NO:12)

Table 5: Antibody 15C3/3 light chain CDRs

CDR	***According to Kabat***
L1	RSSKSLLHSNGNTYLY (SEQ ID NO:13)
L2	RMSNLAS (SEQ ID NO:14)
L3	MQHLEYPLT (SEQ ID NO:15)

Table 6: Antibody 15C3/3 heavy chain CDRs

CDR	***According to Kabat***
H1	SYWMN (SEQ ID NO:16)
H2	QIYPGDGDTNYNGKFKG (SEQ ID NO:17)
H3	VRFDY (SEQ ID NO:18)

The anti-NOGO antibody may also be an antibody which binds to the same epitope on the NOGO polypeptide as an antibody having the CDRs described above. Preferably the epitope comprises the region 586 to 785 (NOGO-A amino acid numbering). More preferably the epitope is comprised within the region 586 to 685 or 686 to 785. Competitive inhibition assays are used for mapping of the epitopes on an antigen.

The anti-NOGO antibody may be a chimeric antibody which binds to and neutralises NOGO, preferably human NOGO, comprising CDRs such as those disclosed in tables 1 to 6. Preferably the chimeric antibody comprises mouse and human sequences.

Preferably the anti-NOGO antibody is a humanised antibody which binds to and neutralises NOGO, preferably human NOGO.

Preferably the anti-NOGO antibody is an antibody or functional fragment thereof which comprises a heavy chain variable domain which comprises one or more CDR's selected from CDRH1, CDRH2 and CDRH3 of table 1, preferably comprising at least CDRH3, and/or a light chain variable domain which comprises one or more CDRs selected from CDRL1, CDRL2 and CDRL3 from table 4; an antibody or functional fragment thereof which comprises a heavy chain variable domain which comprises one or more CDR's selected from CDRH1, CDRH2 and CDRH3 of table 2, preferably comprising at least CDRH3, and/or a light chain variable domain which comprises one or more CDRs selected from CDRL1, CDRL2 and CDRL3 from table 5; or an antibody or functional fragment thereof which comprises a heavy chain variable domain which comprises one or more CDR's selected from CDRH1, CDRH2 and CDRH3 of table 3, preferably comprising at least CDRH3, and/or a light chain variable domain which comprises one or more CDRs selected from CDRL1, CDRL2 and CDRL3 from table 6.

More preferably the anti-NOGO antibody or functional fragment thereof comprises:

a) a heavy chain variable domain (V_H) which comprises in sequence CDRH1, CDRH2 and CDRH3 from table 1,

and /or

b) a light chain variable domain (V_L) which comprises in sequence CDRL1, CDRL2 and CDRL3 from table 4;

an anti-NOGO antibody or functional fragment thereof which comprises:

a) a heavy chain variable domain (V_H) which comprises in sequence CDRH1, CDRH2 and CDRH3 from table 2,

and /or

b) a light chain variable domain (V_L) which comprises in sequence CDRL1, CDRL2 and CDRL3 from table 5; or

an anti-NOGO antibody or functional fragment thereof which comprises:

a) a heavy chain variable domain (V_H) which comprises in sequence CDRH1, CDRH2 and CDRH3 from table 3,

and /or

c) a light chain variable domain (V_L) which comprises in sequence CDRL1, CDRL2 and CDRL3 from table 6.

The anti-NOGO antibody or functional fragment thereof may preferably comprise a heavy chain variable region comprising one of the following amino acid sequences:-

QVQLQQPGTELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEWIGNINPSNGGTN
YNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYCELGQGYWGQGTTLTVSS
(SEQ ID NO: 37); or

EVQLVESGGGLVKPGGSLKLSCAASGFTFSCYAMSWVRQTPEKRLEWVASISDGGSYTY
YPDNVKGRFTISRDNAKNNLYLQMSHLKSEDTAMYYCAKELLFDYWGQGTTLTVSS
(SEQ ID NO:38); or

QVQLQQSGAELVKPGASVKISCKASGYAFSSYWMNWVKQRPGKGLEWIGQIYPGDGDTN
YNGKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCAVRFDYWGQGTTLTVSS
(SEQ ID NO:39).

Further, the anti-NOGO antibody or functional fragment thereof may preferably comprise a heavy chain variable region comprising one of the following amino acid sequences:-

DIVITQDELSNPVTSGESVSISCRSSKSLLYKDGKTYLNWFLQRPGQSPQLLIYLMSTR
ASGVSDRFSGSGSGTDFTLEISRVKAEDVGVYYCQQLVEYPLTFGAGTKLELK
(SEQ ID NO:40); or

DVVMTQTPLSLPVSLGDQASISC[RSSQSLVHSNGNTYLH]WYLQKPGQSPKLLIY[KVSNR]
[FS]GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC[SQSTHVPLT]FGAGTKLELK
(SEQ ID NO:41); or

DIVMTQAAPSVPVTPGESVSISC[RSSKSLLHSNGNTYLY]WFLQRPGQSPQLLIY[RMSNL]
[AS]GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC[MQHLEYPLT]FGAGTKLELK
(SEQ ID NO:42).

The boxed sequences in SEQ ID Nos 37 to 42 represent the CDR sequences according to Kabat et al *supra*.

In a preferred embodiment the anti-NOGO antibody comprises:

a) a heavy chain variable region of SEQ ID NO:37 together with a light chain variable region comprising the amino acid sequence of SEQ ID NO:40; or

b) a heavy chain variable region of SEQ ID NO:38 together with a light chain variable region comprising the amino acid sequence of SEQ ID NO:41; or

c) a heavy chain variable region of SEQ ID NO:39 together with a light chain variable region comprising the amino acid sequence of SEQ ID NO:42.

More preferably the anti-NOGO antibody, or functional fragment thereof, comprises either:

a heavy chain variable fragment comprising SEQ ID NO:37 and a constant part or fragment thereof of a human heavy chain; and

a light chain variable fragment comprising SEQ ID No:40 and a constant part or fragment thereof of a human light chain; or

a heavy chain variable fragment comprising SEQ ID NO:38 and a constant part or fragment thereof of a human heavy chain; and

a light chain variable fragment comprising SEQ ID No:41 and a constant part or fragment thereof of a human light chain; or

a heavy chain variable fragment comprising SEQ ID NO:39 and a constant part or fragment thereof of a human heavy chain; and

a light chain variable fragment comprising SEQ ID No:42 and a constant part or fragment thereof of a human light chain.

Still more preferably the anti-NOGO antibody is selected from 2A10/3, 2C4/1 or 15C3/3, preferably the humanised form thereof. Most preferably the anti-NOGO antibody is 2A10/3 or the humanised form thereof.

Anti-NOGO antibody 2A10/3 comprises a heavy chain variable region having the amino acid sequence of SEQ ID NO:37 and a light chain variable region having the amino acid sequence of SEQ ID NO:40.

Anti-NOGO antibody 2C4/1 comprises a heavy chain variable region having the amino acid sequence of SEQ ID NO:38 and a light chain variable region having the amino acid sequence of SEQ ID NO:41.

Anti-NOGO antibody 15C3/3 comprises a heavy chain variable region having the amino acid sequence of SEQ ID NO:39 and a light chain variable region having the amino acid sequence of SEQ ID NO:42.

The anti-NOGO antibody may be prepared using standard methods. In particular the NOGO antibody may be prepared using polynucleotides as described below. For example preferred polynucleotides encoding CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 and CDRL3, as disclosed in tables 1 to 6, are shown below in tables 7 to 12.

Table 7: Antibody 2A10/3 light chain CDRs

CDR	
L1	AGGTCTAGTAAGAGTCTCCTATATAAGGATG GGAAGACATACTTGAAT (SEQ ID NO:19)
L2	TTGATGTCCACCCGTGCATCA (SEQ ID NO:20)
L3	CAACAACTTGTAGAGTATCCGCTCACG (SEQ ID NO:21)

Table 8: Antibody 2A10/3 heavy chain CDRs

CDR	
H1	AGCTACTGGATGCAC (SEQ ID NO:22)

H2	AATATTAATCCTAGCAATGGTGGTACTAACTACAAT GAGAAGTTCAAGAGC (SEQ ID NO:23)
H3	GGACAGGGCTAC (SEQ ID NO:24)

Table 9: Antibody 2C4/1 light chain CDRs

CDR	
L1	AGATCTAGTCAGAGCCTTGTACACAGTAATG GAAACACCTATTTACAT (SEQ ID NO:25)
L2	AAAGTTTCCAACCGATTTTCT (SEQ ID NO:26)
L3	TCTCAGAGTACACATGTTCCG CTCACG (SEQ ID NO:27)

Table 10: Antibody 2C4/1 heavy chain CDRs

CDR	
H1	TTCAGTTGCTATGCCATGTCT (SEQ ID NO:28)
H2	TCCATTAGTGATGGTGGTAGTTACACCTACTATCCA GACAATGTAAAGGGC (SEQ ID NO:29)
H3	GAACTACTTTTTGACTAC (SEQ ID NO:30)

Table 11: Antibody 15C3/3 light chain CDRs

CDR	
L1	AGGTCTAGTAAGAGTCTCCTGCATAGTAATGGCAA CACTTACTTGTAT (SEQ ID NO:31)
L2	CGGATGTCCAACCTTGCCTCA (SEQ ID NO:32)
L3	ATGCAACATCTAGAATATCCGCTCACG (SEQ ID NO:33)

Table 12: Antibody 15C3/3 heavy chain CDRs

CDR	
H1	AGCTACTGGATGAAC (SEQ ID NO:34)
H2	CAGATTTATCCTGGAGATGGTGATACTAACTACAAC GGAAAGTTCAAGGGC (SEQ ID NO:35)
H3	GTACGCTTTGACTAT (SEQ ID NO:36)

Further, preferred polynucleotides encoding the heavy chain variable region comprising the amino acid sequences of SEQ ID NOs 37 to 39 and light chain variable regions comprising the amino acid sequences of SEQ ID NOs 40 to 42 are shown below.

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:37 is

CCAGGTCCAACTGCAGCAGCCTGGGACTGAACTGGTGAAGCCTGGGGCTTCAGTGAAGC
TGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAG
AGGCCTGGACAAGGCCTTGAGTGGATTGGAAATATTAATCCTAGCAATGGTGGTACTAA
CTACAATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGACAAATCCTCCAGCACAG
CCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTGAACTG
GGACAGGGCTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:43)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:38 is:

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTC
CCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTTGCTATGCCA
TGTCTTGGGTTCGCCAGACTCCGGAAAAGAGGCTGGAGTGGGTCGCATCC
ATTAGTGATGGTGGTAGTTACACCTACTATCCAGACAATGTAAAGGGCCG
ATTCACCATCTCCAGAGACAATGCCAAGAACAACCTGTACCTGCAAATGA
GCCATCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAAGGAACTA
CTTTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:44)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:39 is:

CAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAAGCCTGGGGCCTC
AGTGAAGATTTCCTGCAAAGCTTCTGGCTACGCATTCAGTAGCTACTGGA
TGAACTGGGTGAAGCAGAGGCCTGGAAAGGGTCTTGAGTGGATTGGACAG

ATTTATCCTGGAGATGGTGATACTAACTACAACGGAAAGTTCAAGGGCAA
GGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCA
GCAGCCTGACCTCTGAGGACTCTGCGGTCTATTTCTGTGCAGTACGCTTT
GACTATTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:45)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:40 is:

GATATTGTGATAACCCAGGATGAACTCTCCAATCCTGTCACTTCTGGAGA
ATCAGTTTCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTATATAAGGATG
GGAAGACATACTTGAATTGGTTTCTGCAGAGACCAGGACAATCTCCTCAG
CTCCTGATCTATTTGATGTCCACCCGTGCATCAGGAGTCTCAGACCGGTT
TAGTGGCAGTGGGTCAGGAACAGATTTCACCCTGGAAATCAGTAGAGTGA
AGGCTGAGGATGTGGGTGTGTATTACTGTCAACAACTTGTAGAGTATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:46)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:41 is:

GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGA
TCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATG
GAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAG
CTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTT
CAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGG
AGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAGAGTACACATGTTCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:47)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:42 is:

GATATTGTGATGACTCAGGCTGCACCCTCTGTACCTGTCACTCCTGGAGA
GTCAGTATCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTGCATAGTAATG
GCAACACTTACTTGTATTGGTTCCTGCAGAGGCCAGGCCAGTCTCCTCAG
CTCCTGATATATCGGATGTCCAACCTTGCCTCAGGAGTCCCAGACAGGTT
CAGTGGCAGTGGGTCAGGAACTGCTTTCACACTGAGAATCAGTAGAGTGG
AGGCTGAGGATGTGGGTGTTTATTACTGTATGCAACATCTAGAATATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA

(SEQ ID NO:48)

The method of the invention may be carried out *in-vitro* or *in-vivo*. When carried out *in-vitro* the method may be a model for amyloid deposition, APP processing, or BACE activity or it may provide a means to screen for compounds useful in the treatment of neurolgical diseases such as Alzheimer's disease.

When carried out *in-vivo* the method of the invention may be a method of prophylaxis or treatment of neurological diseases/disorders including, but not limited to, acute diseases such as stroke, traumatic brain injury and spinal cord injury as well as chronic diseases including Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis and inclusion body myositis.

In a preferred embodiment the method is a method of prophylaxis or treatment of neurological disease that is involving amyloidosis. In a more preferred embodiment the amyloidosis is precipitated by an amyloidogenic peptide derived from APP, preferably Aβ; that is Aβ40, Aβ42 or both Aβ40 and Aβ42. Still more preferably the invention relates to a method of prophylaxis or treatment of Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis or inclusion body myositis. Most preferably the invention relates to a method of prophylaxis or treatment of Alzheimer's disease.

The method of prophylaxis or treatment comprises the use of a therapeutic agent or pharmaceutical composition in the treatment of the disease as described hereinbelow.

In another aspect the invention provides the use of a NOGO antagonist in the manufacture of a medicament for the prophylaxis or treatment of neurological diseases/disorders including, but not limited to, acute diseases such as stroke, traumatic brain injury and spinal cord injury as well as chronic diseases including Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis and inclusion body myositis.

In a preferred embodiment the invention provides the use of NOGO antagonist for the manufacture of a medicament for the prophylaxis or treatment of a neurological disease that is involving amyloidosis. In a more preferred embodiment the amyloidosis is precipitated by an amyloidogenic peptide derived from APP, preferably Aβ; that is Aβ40, Aβ42 or both Aβ40 and Aβ42. Still more preferably the invention relates to the use of a NOGO antagonist for the manufacture of a medicament for the prophylaxis or treatment of Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis or inclusion body myositis. Most preferably the invention relates to the use of a NOGO antagonist for the manufacture of a medicament for the prophylaxis or treatment of Alzheimer's disease.

The NOGO antagonist for use in the method of the invention, including the method of treatment or the use for the manufacture of a medicament as hereinabove defined, is preferably a NOGO-A antagonist, most preferably a monoclonal antibody.

Preferably the monoclonal antibody is a function-blocking anti-NOGO-A antibody as described hereinabove. An example of such a preferred function-blocking anti-NOGO-A antibody is 2A10/3 as described hereinabove, or most preferably a humanised version thereof. Other examples are 2C4/1 and 15C3/3 or humanised versions thereof.

The NOGO antagonist may be administered as a prophylactic or post injury, or as otherwise needed. The dose and duration of treatment relates to the relative duration of the molecules of the present invention in the human circulation, and can be adjusted by one of skill in the art depending upon the condition being treated and the general health of the patient.

The mode of administration of the NOGO antagonist may be any suitable route which delivers the agent to the host. The NOGO antagonists and pharmaceutical compositions thereof are particularly useful for parenteral administration, i.e., subcutaneously, intrathecally, intraperitoneally, intramuscularly, intravenously, or intranasally.

NOGO antagonists may be prepared as pharmaceutical compositions containing an effective amount of the NOGO antagonist as an active ingredient in

a pharmaceutically acceptable carrier. In the prophylactic agent of the invention, an aqueous suspension or solution containing the antagonist, preferably buffered at physiological pH, in a form ready for injection is preferred. The compositions for parenteral administration will commonly comprise a solution of the antagonist or a cocktail thereof dissolved in an pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, e.g., 0.9% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques (e.g., filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the antagonist in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and between about 1 ng to about 100 mg, e.g. about 50 ng to about 30 mg or more preferably, about 5 mg to about 25 mg, of a NOGO antagonist. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain about 250 ml of sterile Ringer's solution, and about 1 to about 30 and preferably 5 mg to about 25 mg of an antagonist. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

It is preferred that the NOGO antagonist, when in a pharmaceutical preparation, be present in unit dose forms. The appropriate therapeutically effective dose can be determined readily by those of skill in the art. To effectively treat neurological diseases in a human, one dose of up to 700 mg per 70 kg body weight of an antagonist should be administered parenterally, preferably *i.v.* or i.m. (intramuscularly). Such dose may, if necessary, be repeated at appropriate time intervals selected as appropriate by a physician.

The NOGO antagonists described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. Where the NOGO antagonist is an antibody this technique has been shown to be effective with conventional immunoglobulins and art-known lyophilization and reconstitution techniques can be employed.

The following examples illustrate the invention.

Examples

Example 1 – Transfection of NOGO A cDNA into SHSY5Y-APP cells

The day before transfection the SHSY5Y-APPwt cells were trypsinised, counted and replated at 1million cells per well of a 6-well plate (Nunc).

The NOGO expression construct (FLAG-tagged NOGO-A cDNA in pCDNA3 (Invitrogen); MYC-tagged NOGO-B and NOGO-C in pCDNA3.1A) was complexed with Plus™ reagent by diluting the DNA into serum free medium (OptiMEM-1), adding Plus™ reagent, mixing and incubating at room temperature for 15 min. (6ul Plus reagent into a total volume of 100ul with 2ug DNA and OptiMEM-1 per well)

LipofectAMINE™ reagent was diluted into serum free medium (Optimem-I) in a second tube and mixed (4ul Lipofectamine in 100ul volume per well).

Pre-complexed DNA (from above) was combined with the diluted lipofectAMINE, mixed and incubated at room temp for 15 min.

Meanwhile the cells were washed with serum free medium (OptiMEM-I) and then fresh serum free medium was added to the cells (800ul per well).

The DNA-Plus-LipofectAMINE reagent complexes were then added to the cells (200ul), mixed gently and the cells incubated at 37oC for 5hr in 5% CO2.

After 5 hours 1ml serum containing growth medium was added to the cells and the cells incubated overnight or 2hours.

After 14 hours (or 2 hours) all medium was removed and replaced with 1ml (OptiMEM-1) per well.

After 48 hours medium conditioning the medium was collected and assayed for amyloid content as described in example 2.

Example 2 – Detection of Aβ peptide by IGEN ELISA

SHSY5Y cells overexpressing the human APPwt or Amyloid Precursor Protein Swedish variant sequence (APPswe) were seeded in 96 well Nunc plates at a density of $1x10^5$ cells/well.

After 24 hours the reagents (eg. antibody, peptides, compounds etc) for testing were added to the cells in a final volume of 120µl and cells incubated for 24hr. The medium was removed from cells and 50ul was assayed for Aβ x-40 and 50µl for Aβ x-42 in an overnight ORIGEN immunoassay employing Aβ C-terminal specific antibodies. Briefly, Aβ peptides were captured using biotinylated 6E10 (Signet Labs). Ori-tagged labelled Aβ C-terminal specific antibodies were used to detect the Aβ x-40 and Aβ x-42 species. Antibody- Aβ complexes were captured with streptavidin coated dynabeads and assayed in an IGEN M8 analyser.

The viabiliy of the cells was checked using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) reagent. Briefly, MTT reagent (5mg/ml in phosphate buffered saline) was diluted 1:10 in culture medium and 100µl added to each well. Following incubation at 37°C for 4hr, 100µl solubilising solution (20% SDS/50% Dimethyl Formamide) was added. Absorbance of plates was read using a Delfia Wallac plate reader at 590nM.

The results are shown in figures 1 to 14.

SHSY5Y-APPwt cells express wild-type APP. SHSY5Y-APPswe cells express the Swedish mutation form of APP.

Example 3 - The effect of NOGO-A expression on levels of secreted Aβ 40 and Aβ 42 peptide

When an expression construct expressing NOGO-A is introduced into either SHSY5Y-APPwt cells or SHSY5Y-APPswe cells the levels of Aβ 40 and Aβ 42 are seen to significantly increase suggesting that NOGO-A is in some way modulating, directly or indirectly, the proteolyic processing of APP and/ or degradation of Aβ peptides. The fact that the product of this altered APP processing is the peptide Aβ40 could suggest that the effect of NOGO could be at the level of modulating β-secretase activity.

Figure 1 shows the increase in the level of secreted Aβ40 when NOGO-A is expessed from an expression vector. The two left-had bars are the controls (vector alone and vector carrying a control protein, green fluorescent protein (GFP)), showing the backgound levels of Aβ40 production in this cell-line. The remaining bars shows that significantly increased level of Aβ40 peptide are detected when NOGO-A, fused to a FLAG peptide, is expressed in the cells and also that a similar elevation, albeit less marked, when NOGO-B, fused to myc, is expressed.

The same experiment was repeated using an ELISA specific for Aβ42. The results showed a similar elevation in levels of Aβ 42 peptide secretion as that seen in the earlier experiment using Aβ 40 ELISA. Again NOGO-B also showed increased secretion of Aβ 42 peptide and again the increase was less marked than with NOGO-A. The results are shown in figure 2.

Repeat experiments comparing levels of secreted peptide from cells transfected with NOGO-A compared to vector pCDNA3 alone, are shown in figures 3 (for Aβ 40) and 4 (for Aβ 42).

A direct comparison of levels of Aβ 40 and Aβ 42 peptide secreted is shown in figure 5.

Example 4 – Preparation of an anti-NOGO antibodies.

Preparation and selection of the hybridomas

Anti-NOGO monoclonal antibodies are produced by hybridoma cells, the result of the fusion of mouse myeloma cells with B lymphocytes from mice immunised with the target antigen. The hybridoma cell is immortalised by the myeloma fusion partner while the capacity to produce antibodies is provided by the B lymphocyte. Each hybridoma cell makes only one individual antibody with unique specificity hence the term monoclonal.

SJL mice were immunised with 10ug total protein (1:1, human NOGO-A splice (amino acids 186-1004) and rat NOGO-A splice (amino acids 173-975), produced as GST-fusion proteins in E coli BL21) using both CFA and RIBI adjuvants subcutaneously. The mice were then boosted with 5ug of the same proteins using RIBI adjuvant after 4 and 8 days. After a further 3 days, immune cells were harvested from the locally draining lymph nodes and fused with mouse

myeloma cells using PEG1500 to generate hybridomas. Individual hybridoma cell lines were cloned by two rounds of limiting dilution.

Initial hybridoma antibody selection was on the basis of direct binding to the NOGO protein(s) on microtitre plates. Subsequently ca. 60 hybridomas were selected based on the ability of soluble protein (consisting of human Nogo-A sequence cleaved from the GST moiety using Precission protease) to compete for this binding activity in ELISA assays.

Cloning of the variable regions

Total RNA was extracted from the selected 2A10/3, 2C4/1 and 15C3/3 hybridoma cells followed by reverse transcription and polymerase chain reaction (RT-PCR) to extract heavy and light variable-domain cDNA sequence. The forward primer for RT-PCR was a mixture of degenerate primers specific for murine immunoglobulin gene leader-sequences and the reverse primer was an isotype-specific antibody directed to the constant regions. PCR primers were designed to carry 5' restriction enzyme recognition sites to enable cloning into pUC19 for DNA sequencing.

RNA extraction

Total RNA was extracted from pellets of 10^6 cells of each hybridoma clone using the SV Total RNA Isolation System from Promega according to manufacturer's instructions.

Reverse transcription

RNA was reverse transcribed to produce cDNA of the variable heavy and light domains using forward primers specific for the murine leader sequences and reverse primers to murine IgGκ constant regions. The IgGγ1 reverse primer was used for hybridomas 2C4/1 and 15C3/3; and the IgGγ2b for 2A10/3. Forward primers carry a SalI restriction enzyme recognition site at the 5' end, with four extra nucleotides added 5'to this for efficient restriction digestion. These primers were adapted from Jones ST and Bendig MM 1991 (Biotechnology 9, 88-89). Reverse primers carry a XmaI restriction enzyme recognition site plus and extra four nucleotides at the 5' ends.

Primers:

Murine V_H leader sequence forward primers:

AG77: 5'-ACT AGT CGA CAT GAA ATG CAG CTG GGT CAT STT CTT C-3'

AG78: 5'-ACT AGT CGA CAT GGG ATG GAG CTR TAT CAT SYT CTT-3'

AG79: 5'-ACT AGT CGA CAT GAA GWT GTG GTT AAA CTG GGT TTT T-3'

AG80: 5'-ACT AGT CGA CAT GRA CTT TGG GYT CAG CTT GRT TT-3'

AG81: 5'-ACT AGT CGA CAT GGA CTC CAG GCT CAA TTT AGT TTT CCT T-3'

AG82: 5'-ACT AGT CGA CAT GGC TGT CYT RGS GCT RCT CTT CTG C-3'

AG83: 5'-ACT AGT CGA CAT GGR ATG GAG CKG GRT CTT TMT CTT-3'

AG84: 5'-ACT AGT CGA CAT GAG AGT GCT GAT TCT TTT GTG-3'

AG85: 5'-ACT AGT CGA CAT GGM TTG GGT GTG GAM CTT GCT ATT CCT G-3'

AG86: 5'-ACT AGT CGA CAT GGG CAG ACT TAC ATT CTC ATT CCT G-3'

AG87: 5'-ACT AGT CGA CAT GGA TTT TGG GCT GAT TTT TTT TAT TG-3'

AG89: 5'-ACT AGT CGA CAT GAT GGT GTT AAG TCT TCT GTA CCT G-3'

Murine V_L leader sequence forward primers:

AG90: 5'-ACT AGT CGA CAT GAA GTT GCC TGT TAG GCT GTT GGT GCT G-3'

AG91: 5'-ACT AGT CGA CAT GGA GWC AGA CAC ACT CCT GYT ATG GGT-3'

AG92: 5'-ACT AGT CGA CAT GAG TGT GCT CAC TCA GGT CCT GGC GTT G-3'

AG93: 5'-ACT AGT CGA CAT GAG GRC CCC TGC TCA GWT TYT TGG MWT CTT G-3'

AG94: 5'-ACT AGT CGA CAT GGA TTT WCA GGT GCA GAT TWT CAG CTT C-3'

AG95: 5'-ACT AGT CGA CAT GAG GTK CYY TGY TSA GYT YCT GRG G-3'

AG96: 5'-ACT AGT CGA CAT GGG CWT CAA GAT GGA GTC ACA KWY YCW GG-3'

AG97: 5'-ACT AGT CGA CAT GTG GGG AYC TKT TTY CMM TTT TTC AAT TG-3'

AG98: 5'-ACT AGT CGA CAT GGT RTC CWC ASC TCA GTT CCT TG-3'

AG99: 5'-ACT AGT CGA CAT GTA TAT ATG TTT GTT GTC TAT TTC T-3'

AG100: 5'-ACT AGT CGA CAT GGA AGC CCC AGC TCA GCT TCT CTT CC-3'

MKV12: 5'-ACT AGT CGA CAT GAA GTT TCC TTC TCA ACT TCT GCT C-3'

Murine γ1 constant region reverse primer:

AG102: 5'-GGA TCC CGG GCC AGT GGA TAG ACA GAT G-3'

Murine γ2b constant region reverse primer:

AG104: 5'-GGA TCC CGG GAG TGG ATA GAC TGA TGG-3'

Murine κ constant region reverse primer:

AG101: 5'-GGA TCC CGG GTG GAT GGT GGG AAG ATG-3'

Pools of murine V_H or V_L leader sequence forward primers were prepared at 50μM. Solutions of the murine γ or κ constant region reverse primers were also prepared at 50μM.

RT-PCR

Reverse transcription of the RNA encoding the variable heavy and light regions was carried out in duplicate using the Access RT-PCR System from Promega according to manufacturer's instructions. Approximately 200ng RNA was included in a 50 µl reaction containing RT-PCR buffer supplied, 0.2 mM dNTPs, 1uM of each primer set, 1uM MgS04 and 5U each of AMV Reverse transcriptase and Tfl DNA polymerase.

RT-PCR cycle:
1- 48°C for 45min
2- 94°C for 2min
3- 94°C for 30sec
4- 50°C for 1min
5- 68°C for 2min
6- 68°C for 7min
steps 3 to 5: repeat 30 times.

pUC19 cloning

The variable region RT-PCR products were purified using a Qiagen MinElute Qiagen PCR Purifcation kit according to their instructions and digested sequentially with XmaI and SalI from New England Biolabs according to manufacturer's instructions. They were then loaded on a preparative 1% agarose gel containing 0.5% ethidium bromide and run in TAE buffer at 50mA for 1hour and the V region bands excised under ultra-violet light. The DNA fragments were purified from the gel using the MinElute Gel extraction kit from Qiagen according to manufacturer's instructions. pUC19 vector arms were prepared by digesting pUC19 with SalI and XmaI, then purified using the MinElute Reaction Clean up kit from Qiagen and dephosphorylated using Shrimp alkaline phosphatase (USB) according to the manufacturer's instructions. The concentration of the vector arms and the V-region fragments was estimated from an analytical 1% agarose/ethidium bromide gel, mixed in a molar ratio of 1:2 and ligated using Promega's Quick Ligation kit acording to the manufacturer's instructions. Ligated plasmids were transformed into DH5a cells (Invitrogen) according manufacturer's instructions. Colonies which grew on L-agar plates containing 100ug/ml ampicillin were selected for DNA sequence analysis.

Variable region sequencing

Colonies were cultured overnight at 37°C in 5ml LB medium supplemented with 100μg/ml ampicillin amd plasmid DNA was extracted and purified using the Qiagen QIAprep Spin Miniprep kit according to manufacturer's instructions. The V_H and V_L regions were DNA sequenced using standard M13 forward and reverse primers.

The results of the sequencing determination are shown as SEQ ID NOs 43 to 48.

Heavy chain variable regions:

2A10/3

CCAGGTCCAACTGCAGCAGCCTGGGACTGAACTGGTGAAGCCTGGGGCTTCAGTG
AAGCTGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGGATGCACTGGG
TGAAGCAGAGGCCTGGACAAGGCCTTGAGTGGATTGGAAATATTAATCCTAGCAA
TGGTGGTACTAACTACAATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGAC
AAATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTG
CGGTCTATTATTGTGAACTGGGACAGGGCTACTGGGGCCAAGGCACCACTCTCAC
AGTCTCCTCA
(SEQ ID NO:43)

QVQLQQPGTELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEWIGNINPS
NGGTNYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYCELGQGYWGQGTT
LTVSS
(SEQ ID NO: 37)

2C4/1

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTC
CCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTTGCTATGCCA
TGTCTTGGGTTCGCCAGACTCCGGAAAAGAGGCTGGAGTGGGTCGCATCC
ATTAGTGATGGTGGTAGTTACACCTACTATCCAGACAATGTAAAGGGCCG
ATTCACCATCTCCAGAGACAATGCCAAGAACAACCTGTACCTGCAAATGA
GCCATCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAAGGAACTA
CTTTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:44)

EVQLVESGGGLVKPGGSLKLSCAASGFTFSCYAMSWVRQTPEKRLEWVASISDG
GSYTYYPDNVKGRFTISRDNAKNNLYLQMSHLKSEDTAMYYCAKELLFDYWGQG
TTLTVSS
(SEQ ID NO:38)

15C3/3

CAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAAGCCTGGGGCCTC
AGTGAAGATTTCCTGCAAAGCTTCTGGCTACGCATTCAGTAGCTACTGGA
TGAACTGGGTGAAGCAGAGGCCTGGAAAGGGTCTTGAGTGGATTGGACAG
ATTTATCCTGGAGATGGTGATACTAACTACAACGGAAAGTTCAAGGGCAA
GGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCA
GCAGCCTGACCTCTGAGGACTCTGCGGTCTATTTCTGTGCAGTACGCTTT
GACTATTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:45)

QVQLQQSGAELVKPGASVKISCKASGYAFSSYWMNWVKQRPGKGLEWIGQIYPG
DGDTNYNGKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCAVRFDYWGQGTT
LTVSS
(SEQ ID NO:39)

Light chain variable regions:

2A10/3

GATATTGTGATAACCCAGGATGAACTCTCCAATCCTGTCACTTCTGGAGA
ATCAGTTTCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTATATAAGGATG
GGAAGACATACTTGAATTGGTTTCTGCAGAGACCAGGACAATCTCCTCAG
CTCCTGATCTATTTGATGTCCACCCGTGCATCAGGAGTCTCAGACCGGTT
TAGTGGCAGTGGGTCAGGAACAGATTTCACCCTGGAAATCAGTAGAGTGA
AGGCTGAGGATGTGGGTGTGTATTACTGTCAACAACTTGTAGAGTATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:46)

DIVITQDELSNPVTSGESVSISCRSSKSLLYKDGKTYLNWFLQRPGQSPQLLIY
LMSTRASGVSDRFSGSGSGTDFTLEISRVKAEDVGVYYCQQLVEYPLTFGAGTK
LELK
(SEQ ID NO:40)

2C4/1

GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGA
TCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATG
GAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAG
CTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTT
CAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGG

AGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAGAGTACACATGTTCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:47)

DVVMTQTPLSLPVSLGDQASISC[RSSQSLVHSNGNTYLH]WYLQKPGQSPKLLIY
[KVSNRFS]GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC[SQSTHVPLT]FGAGTK
LELK
(SEQ ID NO:41)

15C3/3
GATATTGTGATGACTCAGGCTGCACCCTCTGTACCTGTCACTCCTGGAGA
GTCAGTATCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTGCATAGTAATG
GCAACACTTACTTGTATTGGTTCCTGCAGAGGCCAGGCCAGTCTCCTCAG
CTCCTGATATATCGGATGTCCAACCTTGCCTCAGGAGTCCCAGACAGGTT
CAGTGGCAGTGGGTCAGGAACTGCTTTCACACTGAGAATCAGTAGAGTGG
AGGCTGAGGATGTGGGTGTTTATTACTGTATGCAACATCTAGAATATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:48)

DIVMTQAAPSVPVTPGESVSISC[RSSKSLLHSNGNTYLY]WFLQRPGQSPQLLIY
[RMSNLAS]GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC[MQHLEYPLT]FGAGTK
LELK
(SEQ ID NO:42).

The DNA sequences for the light or heavy chain regions are given for the antibodies 2A10/3, 2C4/1 and 15C3/3. Below each DNA sequence is the predicted amino acid sequence encoded by that DNA and the CDRs are shown in boxes.

Recombinant anti-NOGO antibodies

Recombinant antibodies having murine 2a/k constant regions could be purified from cells transfected with plasmids comprising the light and heavy variable regions cloned onto mouse IgG2a/k constant region gene segments. The cloned murine V regions were amplified by PCR to introduce restriction sites required for cloning into mammalian expression vectors Rld and Rln. Hind III and Spe I sites were designed in frame with the V_H domain to allow cloning into a modified Rld vector containing the mouse γ2a constant region. Hind III and BsiW I sites were

designed in frame the V_L domain and allow cloning into a modified Rln vector containing the mouse κ constant region.

PCR primers

2A10 V_H forward primer:

5'-
ACTCATAAGCTTGCCACCATGGGATGGAGCTGTATCATCCTCTTTTTGGTAG
-3'

V_H reverse primer:

5'-ACTATGACTAGTGTGCCTTGGCCCCAGTAG-3'

V_L forward primer:

5'- ACTCATAAGCTTGCCACCATGAGGTGCTCTCTTCAGTTTCTG -3'

V_L reverse primer:

5'- ACTATGCGTACGTTTCAGCTCCAGCTTGG -3'

PCR was performed using Hercules (Stratagene) according to the manufacturer's instructions in 50µl volume containing approx 10ng of the pUC19 miniprep containing the V-region, 2% DMSO, 400µM dNTPs, 1µM each primer and buffer supplied. PCR was carried out as follows 1-95 °C 2 mins, 2-95°C 1 min, 3-56 °C 1 min, 4-72°C 1 min. Steps 2-4 30 cycles.

Cloning into expression vectors

The PCR products were purified using the MinElute PCR Purification kit from Qiagen according to manufacturer's instructions. The V_H PCR product and Rld (lgG2a) mammalian expression vector were digested Hind III-Spe I. The V_L PCR product and Rln (k) mammalian expression vector were digested Hind III-BsiW I (NEB) according to manufacturer's instructions. Vectors were ligated to inserts in a 1:2 molar ratio using the Promega Quick Ligation kit. Ligation mixes were transfected into DH5a cells and colonies growing on ampicillin selection were grown up and sent for DNA sequence verification.

Sequencing of recombinant anti-NOGO antibody 2A10/3

The sequence of the 2A10 heavy chain between the HindIII and EcoRI cloning sites was determined to be:

AAGCTTGCCACCATGGGATGGAGCTGTATCATCCTCTTTTTGGTAGCAGC
AGCTACAGGTGTCCACTCCCAGGTCCAACTGCAGCAGCCTGGGACTGAAC
TGGTGAAGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTAC
ACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCCTGGACAAGG
CCTTGAGTGGATTGGAAATATTAATCCTAGCAATGGTGGTACTAACTACA
ATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGACAAATCCTCCAGC
ACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTA
TTATTGTGAACTGGGACAGGGCTACTGGGGCCAAGGCACACTAGTCACCG
TCTCCTCAGCCAAAACAACAGCCCCATCGGTCTATCCACTGGCCCCTGTG
TGTGGAGATACAACTGGCTCCTCGGTGACTCTAGGATGCCTGGTCAAGGG
TTATTTCCCTGAGCCAGTGACCTTGACCTGGAACTCTGGATCCCTGTCCA
GTGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACCCTC
AGCAGCTCAGTGACTGTAACCTCGAGCACCTGGCCCAGCCAGTCCATCAC
CTGCAATGTGGCCCACCCGGCAAGCAGCACCAAGGTGGACAAGAAAATTG
AGCCCAGAGGGCCCACAATCAAGCCCTGTCCTCCATGCAAATGCCCAGCA
CCTAACCTCCTGGGTGGCCCATCCGTCTTCATCTTCCCTCCAAAGATCAA
GGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGG
ATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAAC
GTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAG
TACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGA
GTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCC
ATCGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGT
ATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTC
TGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGG
ACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCT
GGACTCTGATGGTTCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGA
AGAACTGGGTGGAAAGAAATAGCTACTCCTGTTCAGTGGTCCACGAGGGT
CTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAATG
AGAATTC

(SEQ ID NO:49)

The sequence of the 2A10 light chain between the HindIII and EcoRI cloning sites was determined to be:

AAGCTTGCCACCATGAGGTGCTCTCTTCAGTTTCTGGGGGTGCTTATGTT
CTGGATCTCTGGAGTCAGTGGGGATATTGTGATAACCCAGGATGAACTCT
CCAATCCTGTCACTTCTGGAGAATCAGTTTCCATCTCCTGCAGGTCTAGT
AAGAGTCTCCTATATAAGGATGGGAAGACATACTTGAATTGGTTTCTGCA
GAGACCAGGACAATCTCCTCAGCTCCTGATCTATTTGATGTCCACCCGTG
CATCAGGAGTCTCAGACCGGTTTAGTGGCAGTGGGTCAGGAACAGATTTC

ACCCTGGAAATCAGTAGAGTGAAGGCTGAGGATGTGGGTGTGTATTACTG
TCAACAACTTGTAGAGTATCCGCTCACGTTCGGTGCTGGGACCAAGCTGG
AGCTGAAACGTACGGATGCTGCACCGACTGTATCCATCTTCCCACCATCC
AGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAA
CTTCTACCCCAAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAAC
GACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAGC
ACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGGACGAGTATGAACG
ACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCA
TTGTCAAGAGCTTCAACAGGAATGAGTGTTAAGAATTC

(SEQ ID NO:50

Example 5 - anti-NOGO-A antibody inhibits Aβ 40 and Aβ 42 peptide secretion from SHSY5Y-APPwt and SHSY5Yswe cells

Figure 6 shows the dramatic reduction in levels of Aβ 40 and Aβ 42 peptide secreted from SHSY5Y-APPwt cells expressing endogenous NOGO-A when the anti-NOGO antibody 2A10 is introduced into the culture medium.

The effect is seen in a dose-dependent manner, at 30μg/ml reaching inhibition levels of almost 90%. There is no apparent difference in the effect between Aβ 40 and Aβ 42 peptides (white bars and black bars respectively in figure 6), in other words any effect of the anti-NOGO antibody on APP processing is not preferential for either the Aβ 40 or Aβ 42 peptides.

Figure 7 shows the same experiment as in figure 6 but with an unrelated IgG1 antibody. As can clearly be seen in the figure, an unrelated (non anti-NOGO A) monoclonal antibody has no inhibitory effect on the levels of Aβ 40 and Aβ 42 peptide secretion from the cells.

Figure 8 shows that the same unrelated IgG1 antibody similarly shows little or no inhibitory effect on the levels of Aβ 40 and Aβ 42 peptide secretion from SHSY5Y-APPswe cells expressing NOGO-A.

Similarly figure 9 shows the results of the same experiment as described above for figure 6 but using an anti-NOGO monoclonal antibody which binds to NOGO-A but is not a function-blocker (6D5). The non-function-blocking anti-NOGO-A monoclonal antibody has minimal effect (less than 10%) on the secretion of Aβ 40 and Aβ 42 peptides from SHSY5Y-APPwt cells expressing NOGO-A. This

result suggests that the results shown in figure 6 are a result of the inhibition of NOGO functional activity by the anti-NOGO antibody.

Figure 10 shows the results of the same experiment as for figure 9, using the non-function blocking anti-NOGO monoclonal antibody (6D5) but with SHSY5Y-APPswe cells expressing endogenous NOGO-A. As before (figure 9) there is minimal effect on the levels of Aβ 40 and Aβ 42 being secreted by this cell line, being less than 10% inhibition.

Figure 11 shows the results of an experiment which extends the results of the experiment of figure 6. Figure 11 shows that the concentrate-dependent effect of the inhibitory effect shown by the function-blocking antibody 2A10/3 continues at a higher concentration, a level of greater than 90% inhibition being achieved at an antibody concentration of 50μg/ml.

Figure 12 shows the results of an identical experiment to that of figure 11 except that SHSY5Yswe cells are used. The concentration-dependent inhibitory effect of the antibody 2A10/3 continues to be seen at the higher concentration of 50μg/ml.

Figure 13 shows the effect of a different function-blocking anti-NOGO-A antibody, 2C4, on the secretion of Aβ 40 and Aβ 42 peptides from SHSY5Y-APPwt cells. The results show a concentration-dependent inhibitory effect on the levels of Aβ 40 and Aβ 42 peptide secretion to a level of 36% at a concentration of 20μg/ml. Again the effect is seen on both Aβ 40 and Aβ 42 peptides.

Figure 14 compares the inhibitory effect of the anti-NOGO-A function-blocking antibodies 2A10, 2C4 and 15C3 (at the concentrations shown in the figure) with other control antibodies 10A4, IgG2b and 14D12. The figure shows that the effect on inhibition of Aβ 40 and Aβ 42 secretion is specific to the function-blocking anti-NOGO-A monoclonal antibodies.

Claims

1. A method of modulating production of an amyloidogenic peptide comprising contacting a cell which is expressing:
a) the precursor from which the amyloidogenic peptide is derived; and
b) a NOGO polypeptide
with a NOGO antagonist.

2. A method according to claim 1 wherein the precursor is APP.

3. A method according to claim 1 or 2 wherein the amyloidogenic peptide is Aβ.

4. A method according to any one of claims 1 to 3 wherein the NOGO polypeptide is NOGO-A.

5. A method according to any one of claims 1 to 4 wherein the NOGO antagonist is a monoclonal antibody.

6. A method according to claim 5 wherein the monoclonal antibody is a function-blocking anti-NOGO-A monoclonal antibody.

7. Use of a NOGO antagonist in the manufacture of a medicament for the treatment or prophylaxis of a disease involving amyloidosis.

8. Use according to claim 7 wherein the amyloidosis is precipitated by an amyloidogenic peptide derived from APP.

9. Use according to claim 8 wherein the amyloidogenic peptide is Aβ.

10. Use according to any one of claims 7 to 9 wherein the disease is Alzheimer's disease.

11. Use according to any one of claims 7 to 10 wherein the NOGO antagonist is a NOGO-A antagonist.

12. Use according to claim 11 wherein the NOGO-A antagonist is a monoclonal antibody.

13. Use according to claim 12 wherein the monoclonal antibody is a function-blocking anti-NOGO-A antibody.

14. Use according to claim 13 wherein the function-blocking anti-NOGO antibody is an antibody which binds to a region of human NOGO between 586 to 785 (NOGO-A amino acid numbering).

15. Use according to claim 12 wherein the anti-NOGO-A antibody comprises one or more of the following CDRs:

Light chain CDRs

CDR	***According to Kabat***
L1	RSSKSLLYKDGKTYLN (SEQ ID NO:1)
L2	LMSTRAS (SEQ ID NO:2)
L3	QQLVEYPLT (SEQ ID NO:3)

Heavy chain CDRs

CDR	***According to Kabat***
H1	SYWMH (SEQ ID NO:4)
H2	NINPSNGGTNYNEKFKS (SEQ ID NO:5)
H3	GQGY (SEQ ID NO:6)

16. Use according to claim 12 wherein the anti-NOGO-A antibody comprises one or more of the following CDRs:

Light chain CDRs

CDR	***According to Kabat***
L1	RSSQSLVHSNGNTYLH (SEQ ID NO:7)
L2	KVSNRFS (SEQ ID NO:8)
L3	SQSTHVPLT (SEQ ID NO:9)

Heavy chain CDRs

CDR	*According to Kabat*
H1	FSCYAMS (SEQ ID NO:10)
H2	SISDGGSYTYYPDNVKG (SEQ ID NO:11)
H3	ELLFDY (SEQ ID NO:12)

17. Use according to claim 12 wherein the anti-NOGO-A antibody comprises one or more of the following CDRs:

Light chain CDRs

CDR	*According to Kabat*
L1	RSSKSLLHSNGNTYLY (SEQ ID NO:13)
L2	RMSNLAS (SEQ ID NO:14)
L3	MQHLEYPLT (SEQ ID NO:15)

Heavy chain CDRs

CDR	*According to Kabat*
H1	SYWMN (SEQ ID NO:16)
H2	QIYPGDGDTNYNGKFKG (SEQ ID NO:17)
H3	VRFDY (SEQ ID NO:18)

18. Use according to claim 12 wherein the monoclonal antibody is a humanised antibody.

19. A method of treatment or prophylaxis of Alzheimer's disease which comprises administering to said human in need thereof an effective amount of an anti-NOGO antibody as defined in any one of claims 13 to 18.

Abstract

Methods of modulating production of an amyloidogenic peptide is disclosed. Use of such methods in the treatment of diseases involving amyloidosis, for example Alzheimer's disease, is also disclosed.

In the figures:

Figure 1: NOGO A transfection leads to elevation of Aβ 40 peptide levels in SHSY5Y-APPwt cells

Figure 2: NOGO A transfection leads to elevation of Aβ 42 peptide levels in SHSY5Y-APPwt cells

Figure 3: Effect of NOGO A expression on Aβ 40 peptide levels

Figure 4: Effect of NOGO A expression on Aβ 42 peptide levels

Figure 5: Effect of NOGO A, NOGO-B and NOGO-C expression on Aβ 40 and Aβ 42

Figure 6. Anti-NOGO A antibody 2A10-BR inhibits Aβ secretion from SHSY5Y-APPwt cells

Figure 7. Effect of Chemicon IgG1 on Aβ secretion from SHSY5Y-APPwt cells

Figure 8. Effect of Chemicon IgG1 on Aβ secretion from SHSY5Y-APPswe cells

Figure 9. Effect of control anti-NOGO (non function-blocking) antibody 6D5 on Aβ secretion from SHSY5Y-APPwt cells

Figure 10. Effect of control anti-NOGO (non function-blocking) antibody 6D5 on Aβ secretion from SHSY5Y-APPswe cells

Figure 11. Function-blocking anti-NOGO A monoclonal antibody 2A10 inhibits Aβ secretion from SHSY5Y-APPwt cells

Figure 12. Function-blocking anti-NOGO A monoclonal antibody 2A10 inhibits Aβ secretion from SHSY5Y-APPswe cells

Figure 13. Function-blocking anti-NOGO A monoclonal antibody 2C4 inhibits Aβ secretion from SHSY5Y-APPwt cells

Figure 14. Effect of anti-NOGO A static culture antibody preparations and additional control antibodies on Aβ secretion from SHSY5Y-APPwt cells. 2A10,

2C4 and 15C3 are the static culture antibodies. All others are BR (Bioreactor) purified controls or commercially available controls.

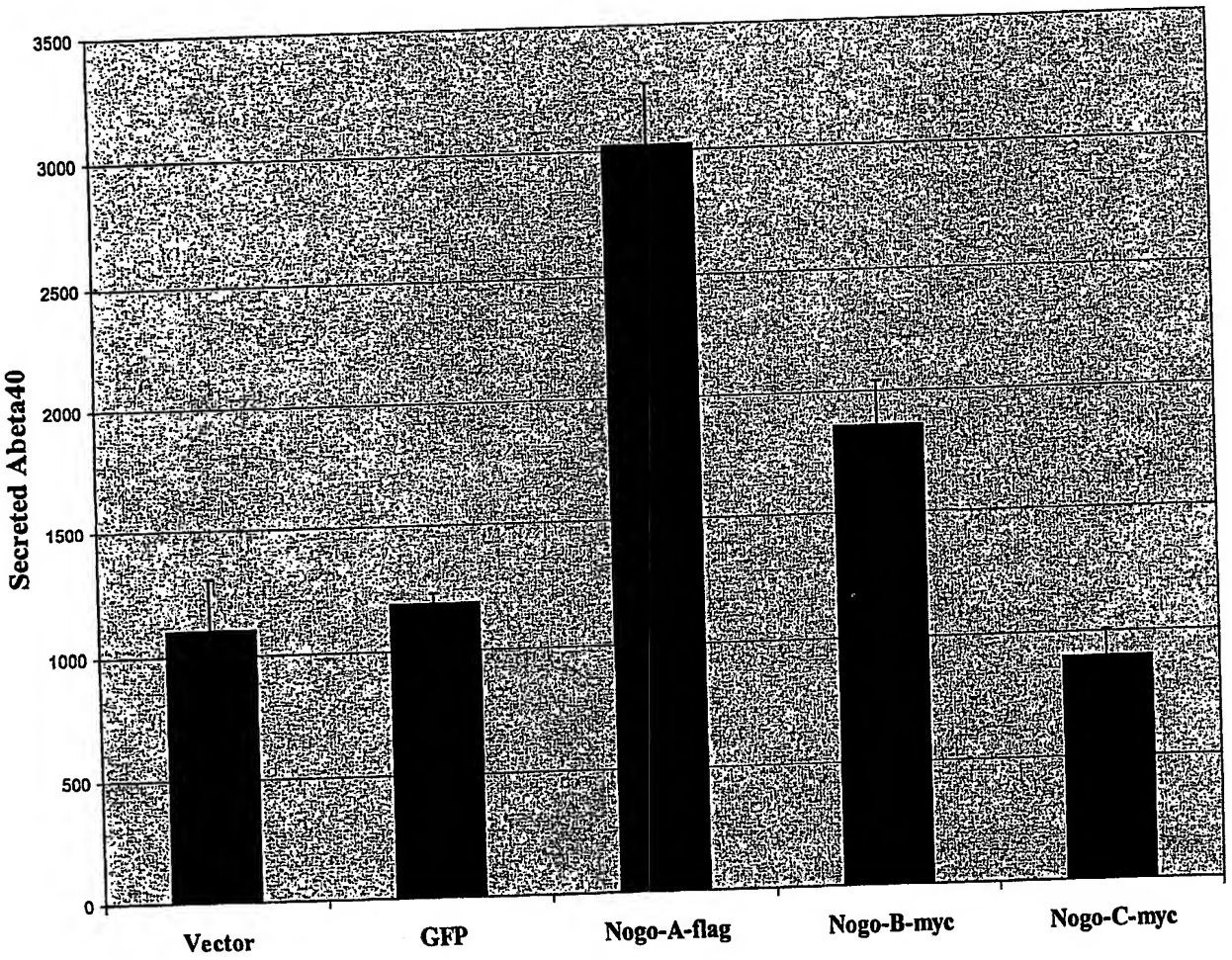

Secreted Abeta40
3500
3000
2500
2000
1500
1000
500
0
Vector
GFP
Nogo-A-flag
Nogo-B-myc
Nogo-C-myc

Figure 1

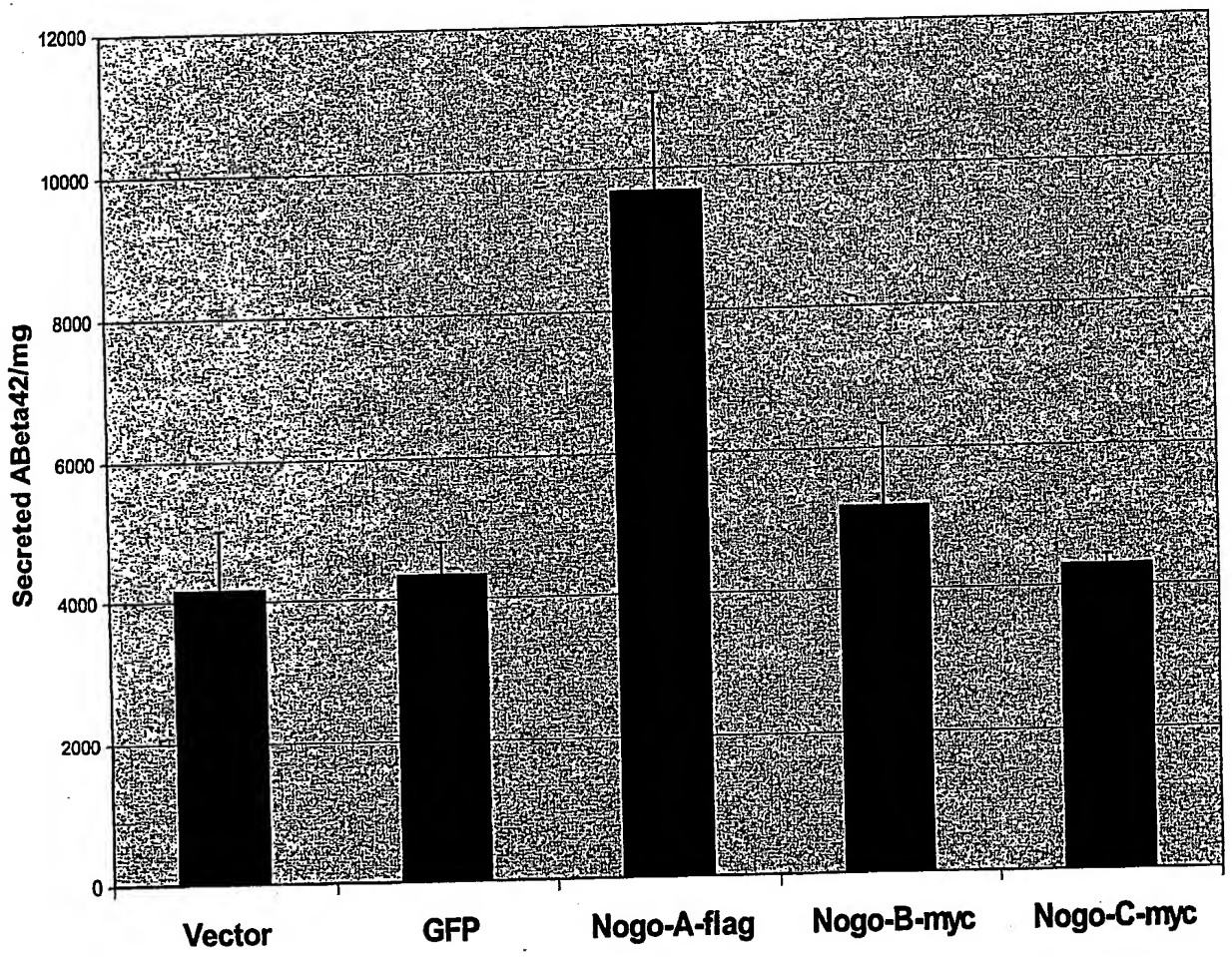

12000
10000
8000
6000
4000
2000
0
Secreted ABeta42/mg
Vector
GFP
Nogo-A-flag
Nogo-B-myc
Nogo-C-myc

Figure 2

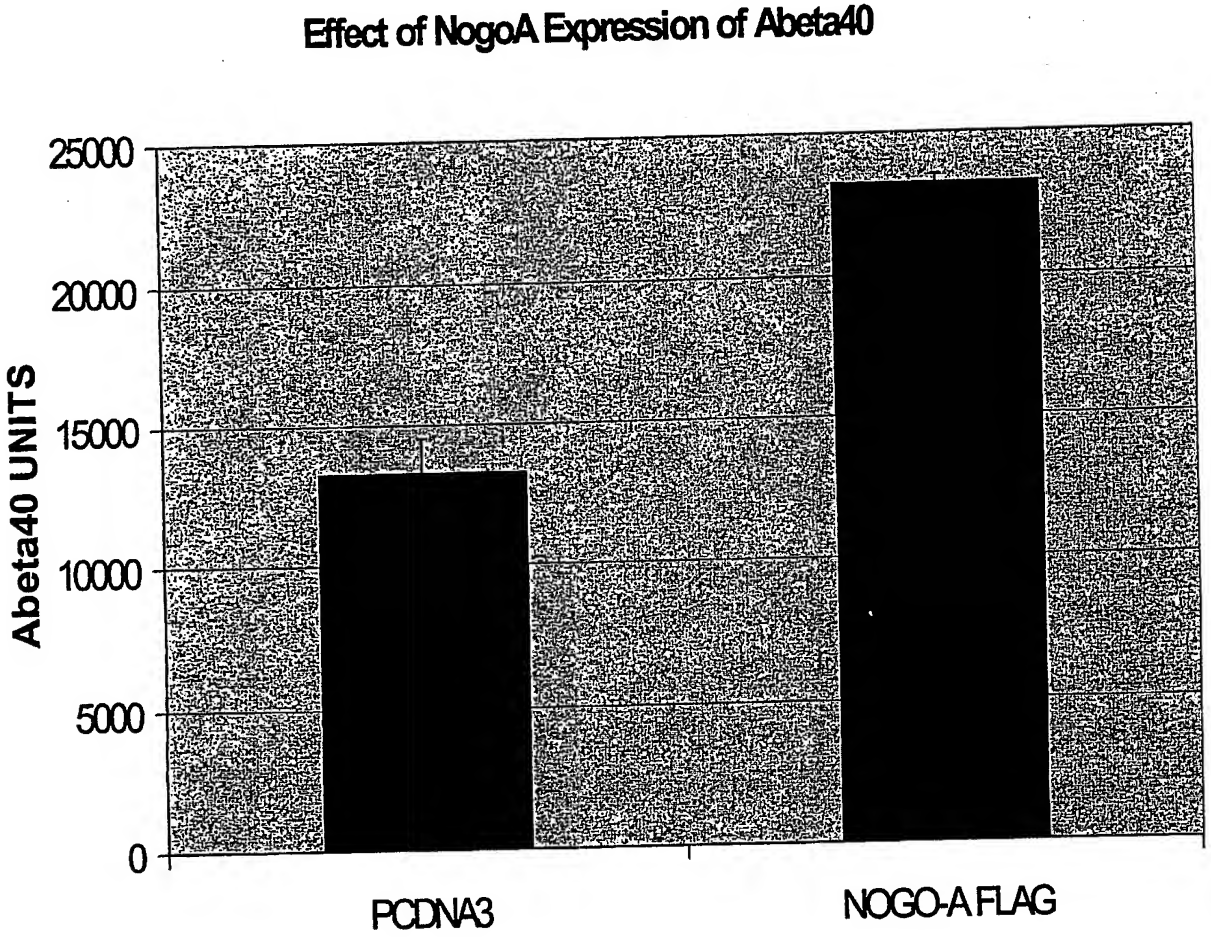

Effect of NogoA Expression of Abeta40
Abeta40 UNITS
25000
20000
15000
10000
5000
0
PCDNA3
NOGO-A FLAG

Figure 3

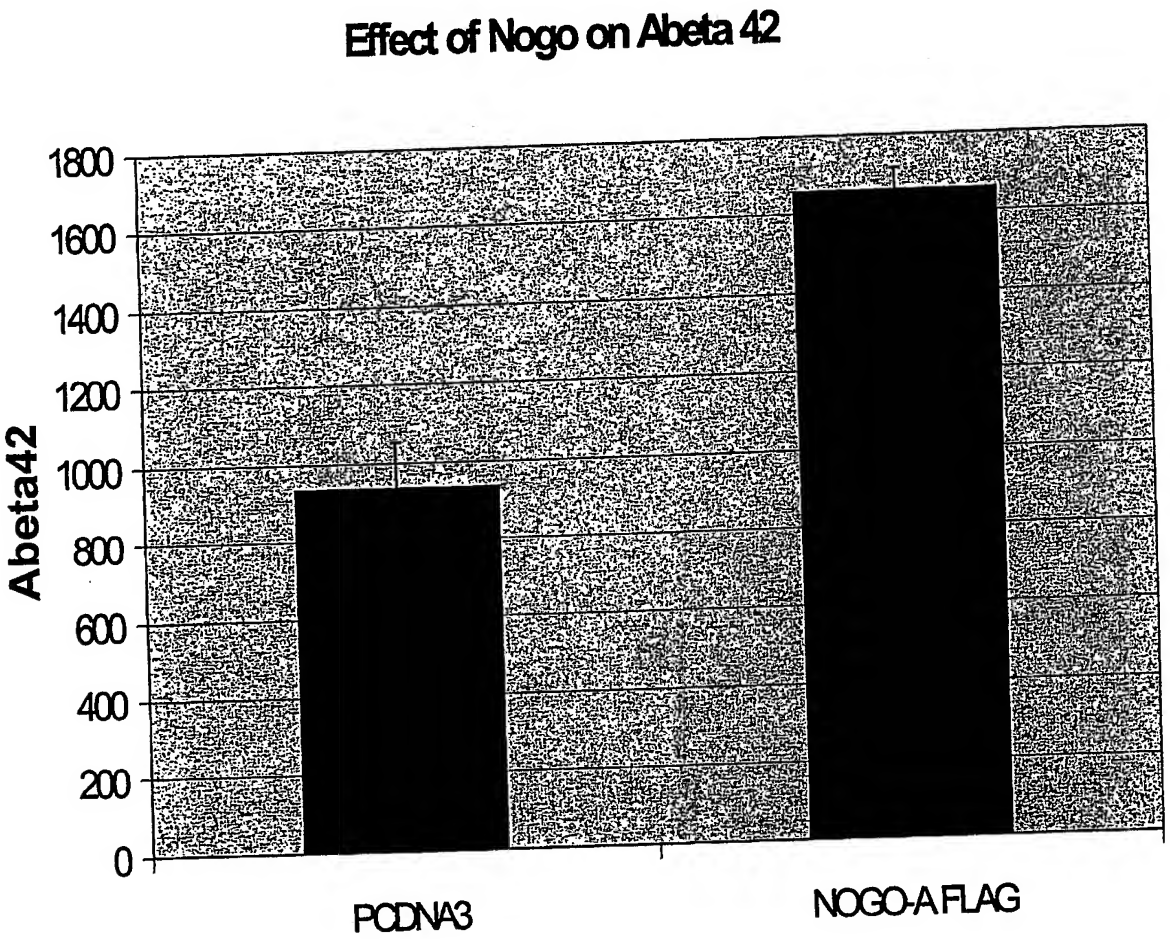

Effect of Nogo on Abeta 42
Abeta42
1800
1600
1400
1200
1000
800
600
400
200
0
PCDNA3
NOGO-A FLAG

Figure 4

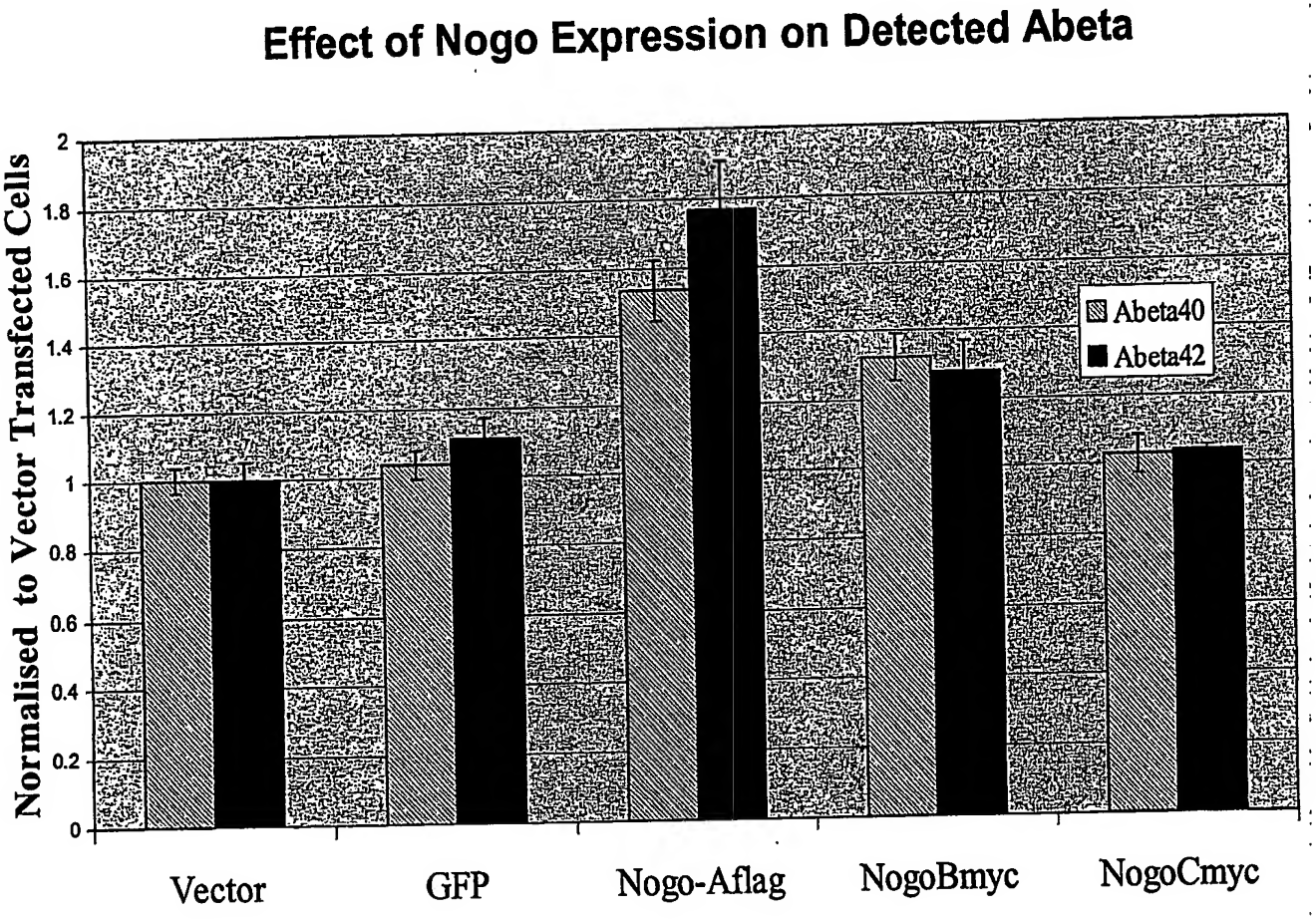

Effect of Nogo Expression on Detected Abeta
Normalised to Vector Transfected Cells
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Abeta40
Abeta42
Vector
GFP
Nogo-Aflag
NogoBmyc
NogoCmyc

Figure 5

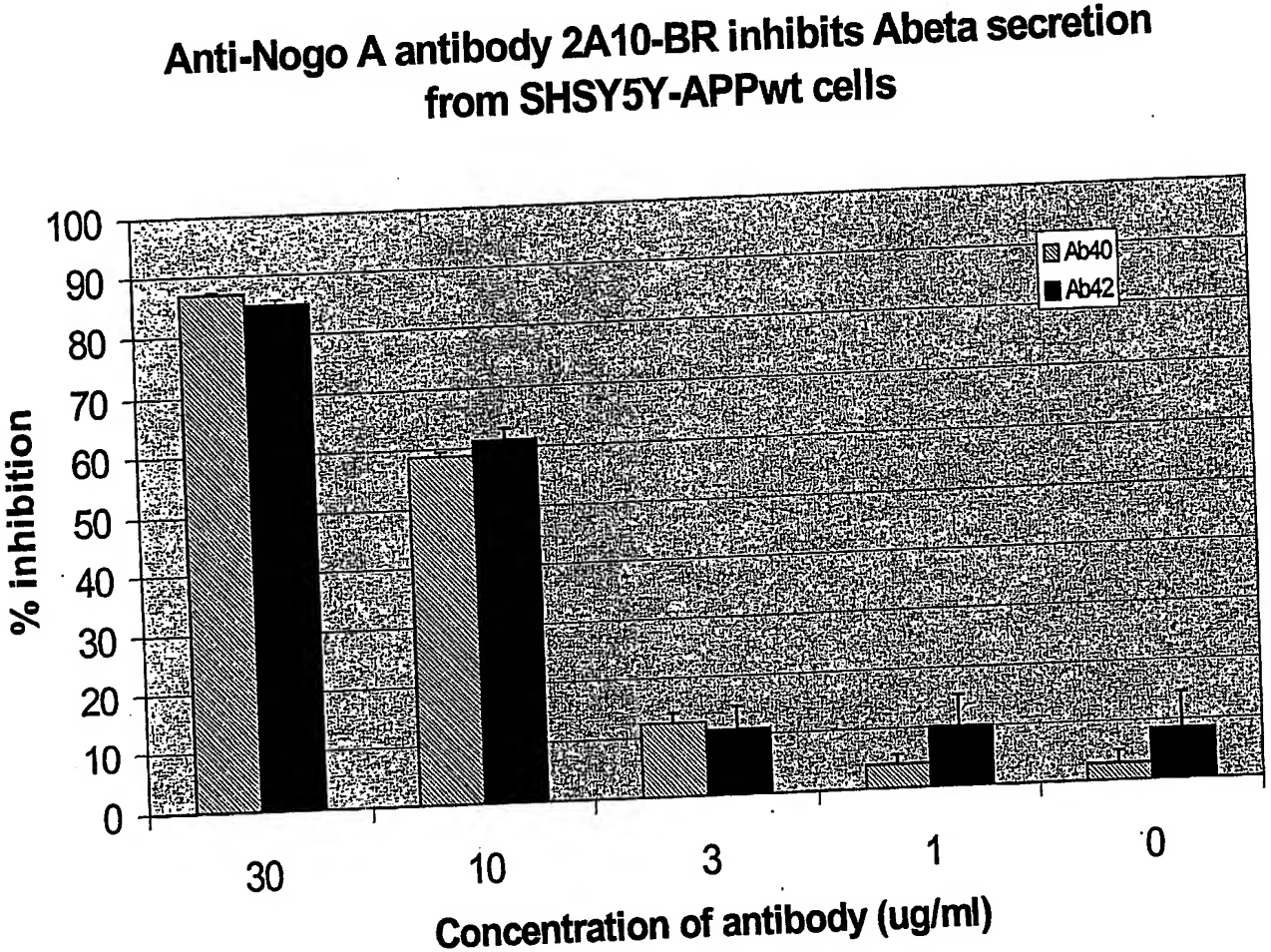

Anti-Nogo A antibody 2A10-BR inhibits Abeta secretion from SHSY5Y-APPwt cells
% inhibition
100
90
80
70
60
50
40
30
20
10
0
Ab40
Ab42
30
10
3
1
0
Concentration of antibody (ug/ml)

Figure 6

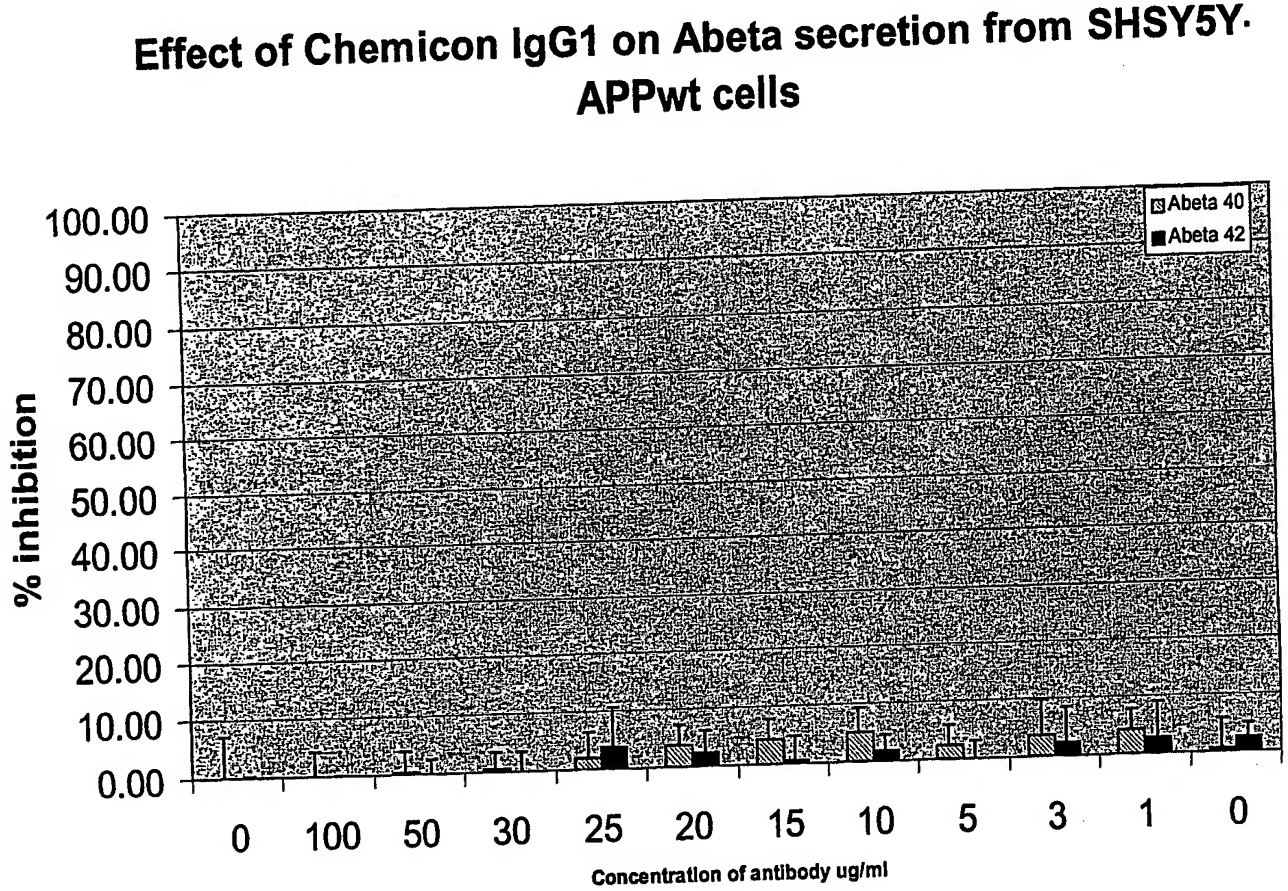

Effect of Chemicon IgG1 on Abeta secretion from SHSY5Y-APPwt cells
Abeta 40
Abeta 42
% inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
100
50
30
25
20
15
10
5
3
1
0
Concentration of antibody ug/ml

Figure 7

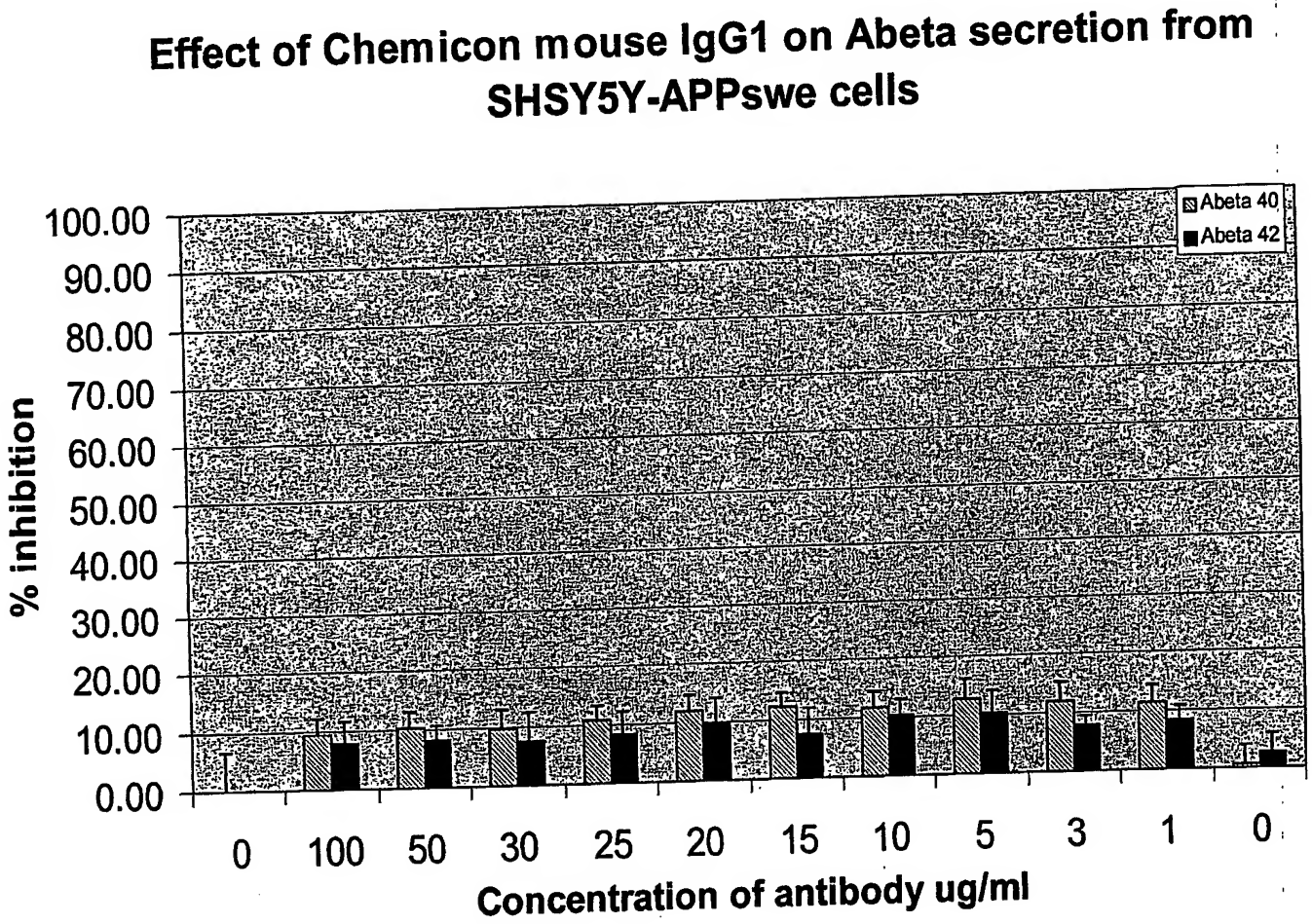

Effect of Chemicon mouse IgG1 on Abeta secretion from SHSY5Y-APPswe cells
Abeta 40
Abeta 42
% inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
100
50
30
25
20
15
10
5
3
1
0
Concentration of antibody ug/ml

Figure 8

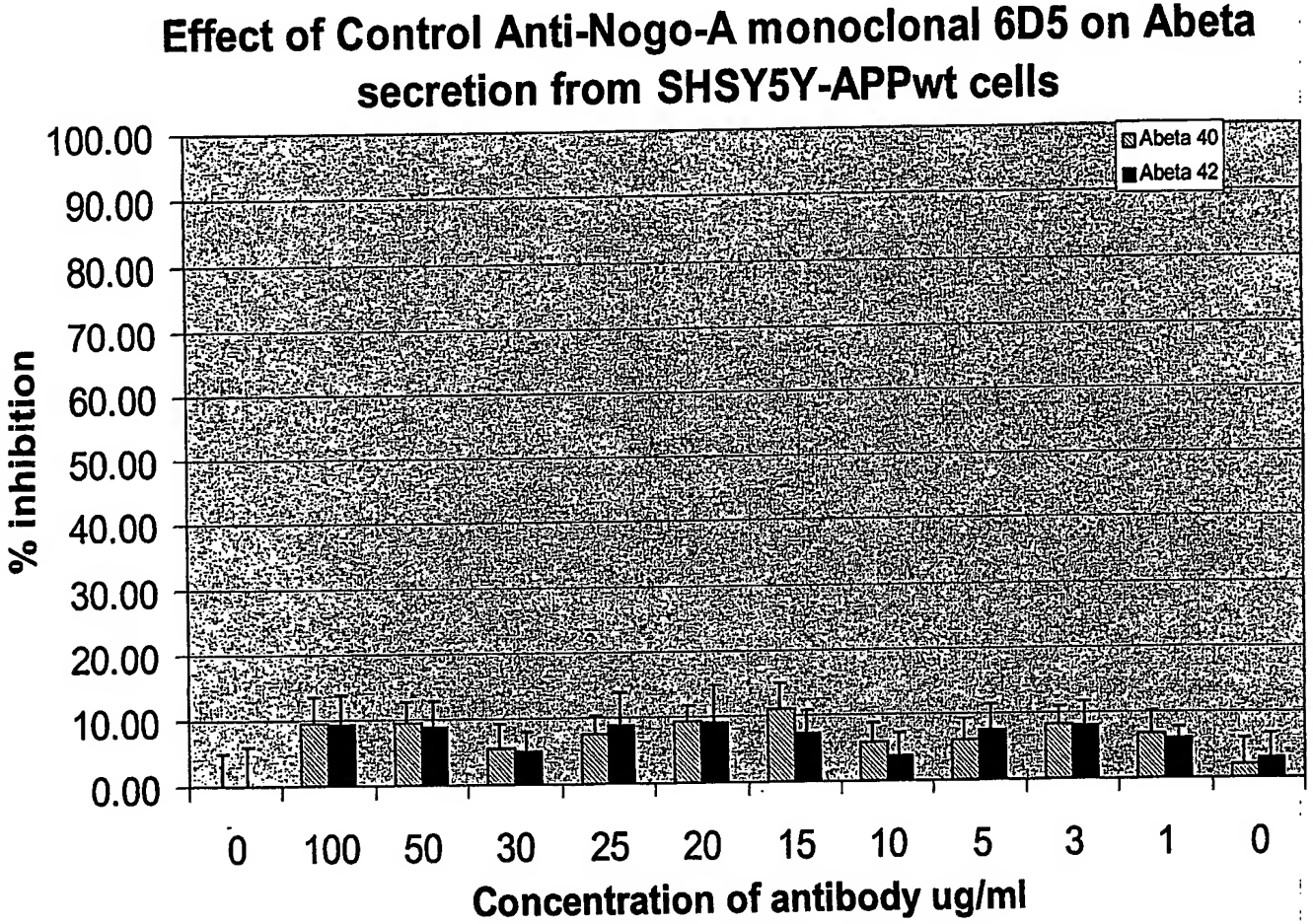

Effect of Control Anti-Nogo-A monoclonal 6D5 on Abeta secretion from SHSY5Y-APPwt cells
Abeta 40
Abeta 42
% inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
100
50
30
25
20
15
10
5
3
1
0
Concentration of antibody ug/ml

Figure 9

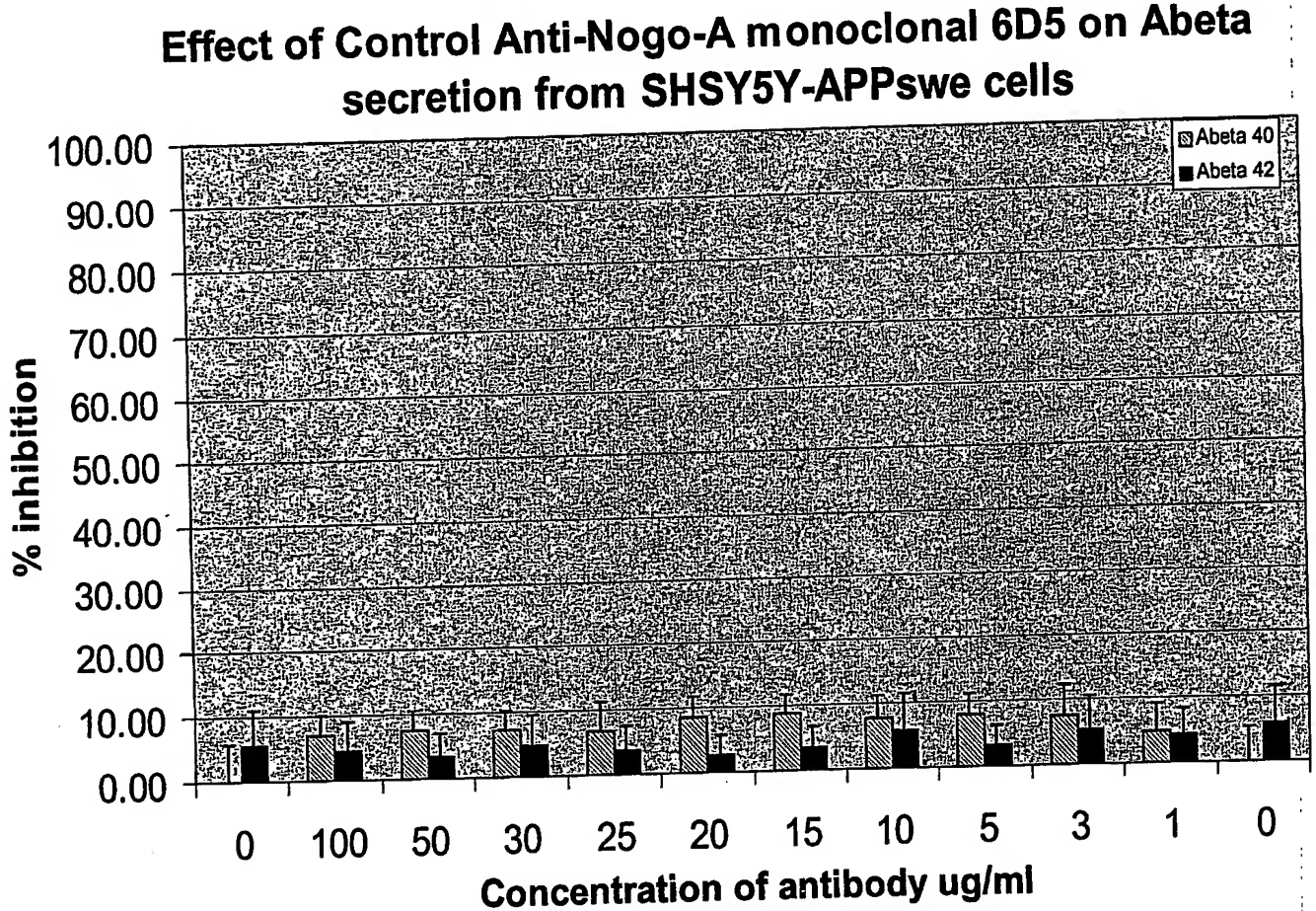

Effect of Control Anti-Nogo-A monoclonal 6D5 on Abeta secretion from SHSY5Y-APPswe cells
Abeta 40
Abeta 42
% inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
100
50
30
25
20
15
10
5
3
1
0
Concentration of antibody ug/ml

Figure 10

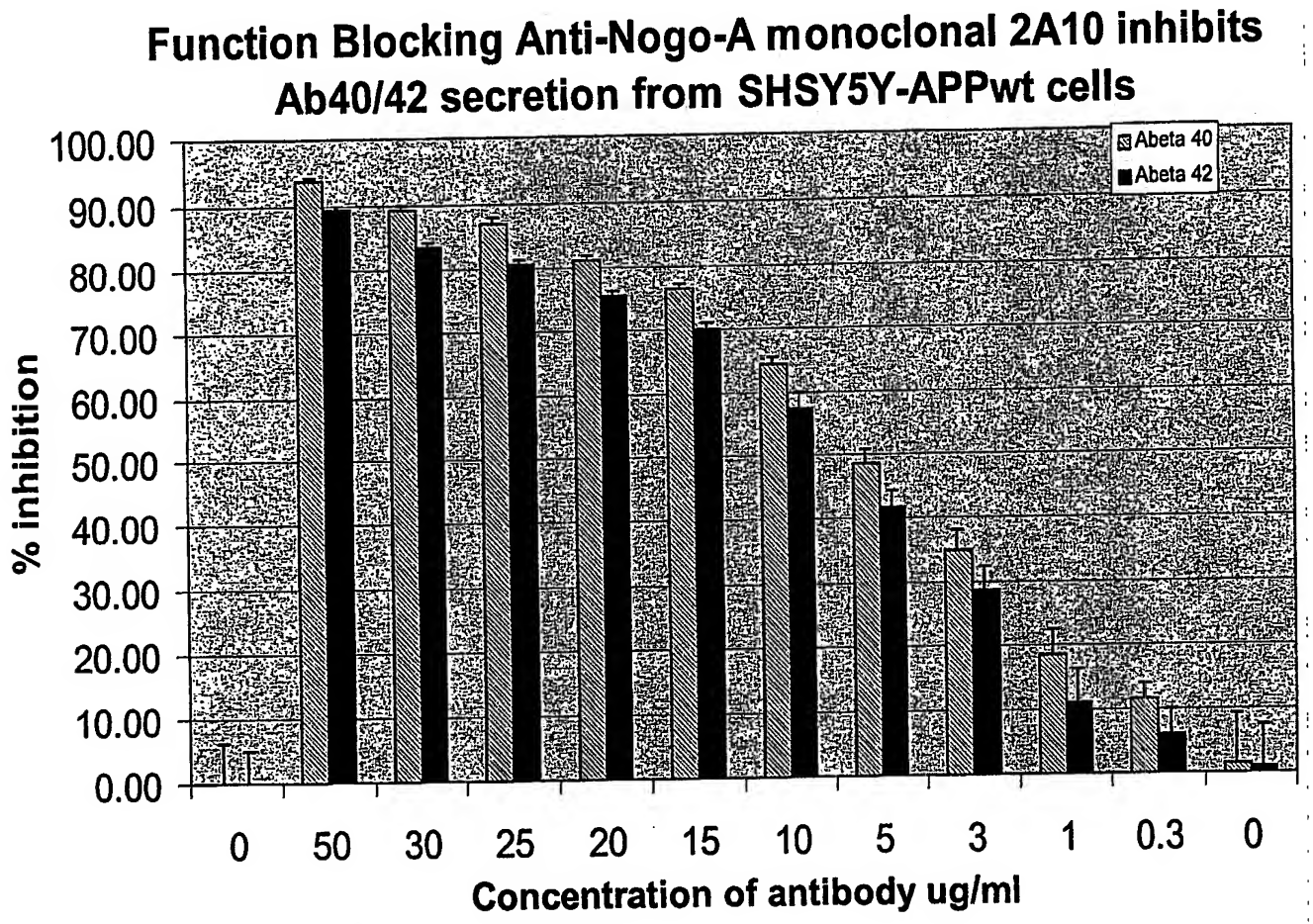

Function Blocking Anti-Nogo-A monoclonal 2A10 inhibits Ab40/42 secretion from SHSY5Y-APPwt cells
Abeta 40
Abeta 42
% inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
50
30
25
20
15
10
5
3
1
0.3
0
Concentration of antibody ug/ml

Figure 11

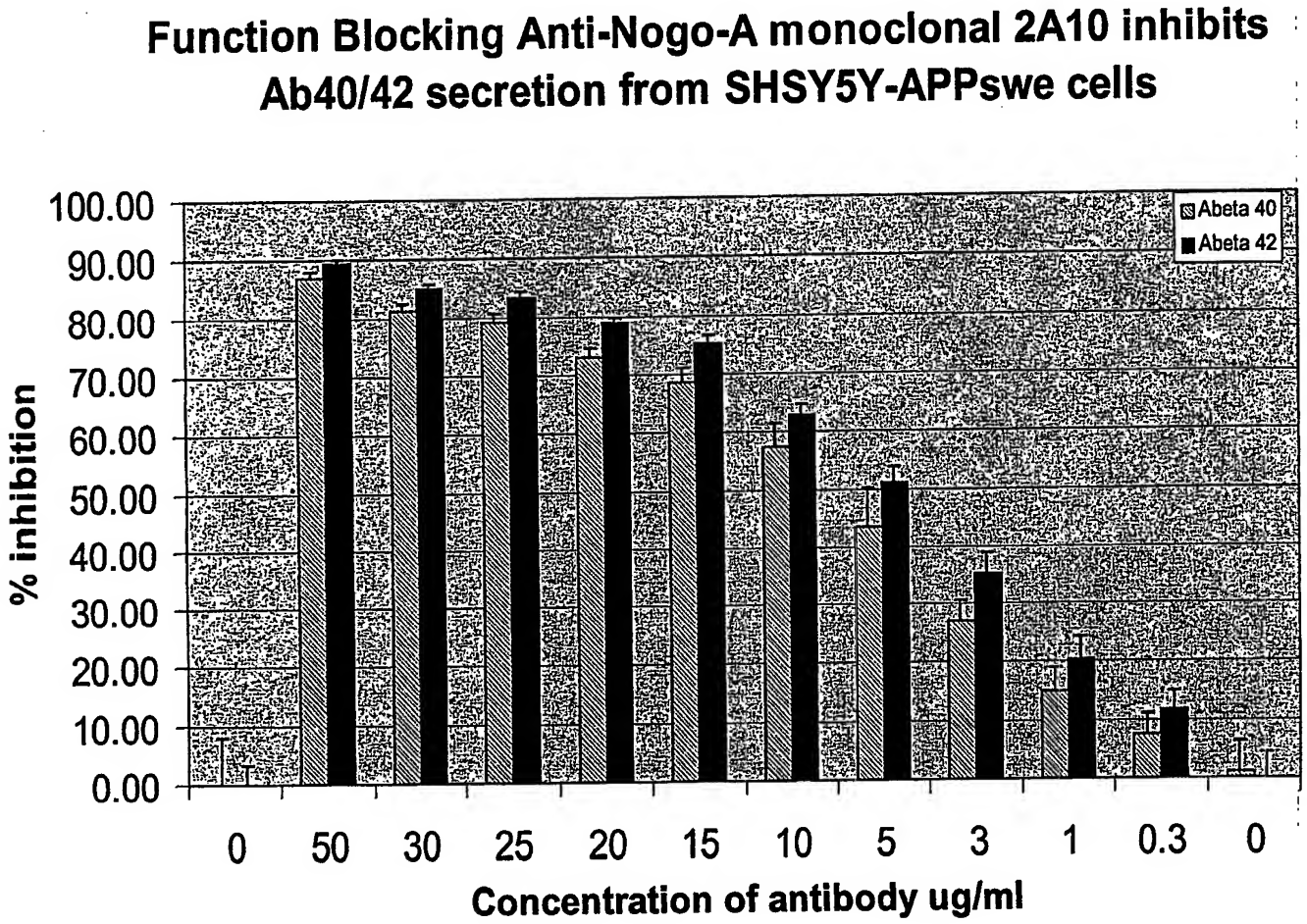

Function Blocking Anti-Nogo-A monoclonal 2A10 inhibits Ab40/42 secretion from SHSY5Y-APPswe cells
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% inhibition
Abeta 40
Abeta 42
0
50
30
25
20
15
10
5
3
1
0.3
0
Concentration of antibody ug/ml

Figure 12

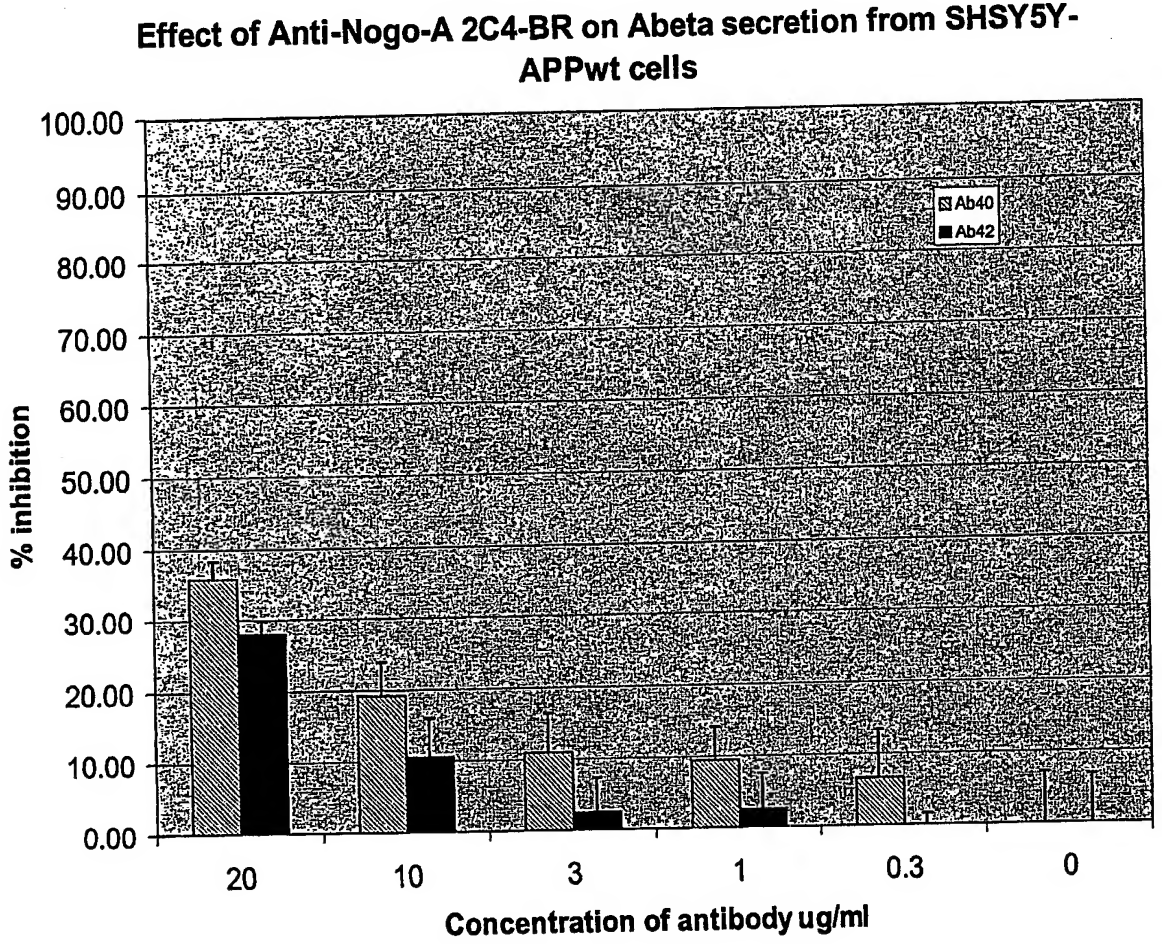

Effect of Anti-Nogo-A 2C4-BR on Abeta secretion from SHSY5Y-APPwt cells
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% inhibition
Ab40
Ab42
20
10
3
1
0.3
0
Concentration of antibody ug/ml

Figure 13

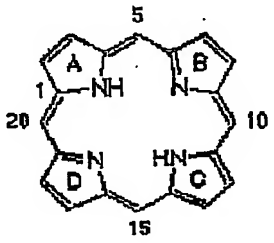
HYPERBRANCHED POLYMERS

This invention relates to hyperbranched polymers, and more particularly to a hyperbranched polymer comprising a porphyrin moiety.

hyperbranched polymers (polymers containing two or more generations of branching) are well known. The formation of high molecular weight hyperbranched polymers from AB_2 monomers containing one group of type A and two of type B was first described in US Patent 4857630. Numerous other hyperbranched polymers have been reported since that time, for example, by Hawker et al, J. Am. Chem. Soc. 113, 4252-4261 (1991); Turner et al, Macromolecules, 27, 1611 (1994); and in US Patents Nos 5196502, 5225522 and 5214122. All of these hyperbranched polymers were obtained by polycondensation processes involving AB_2 monomers.

Topologically, hyperbranched polymers have at least two branching points and one focal point unit or core clearly distinguishable from the end groups. The focal point or core is generally the site of the initiation of the polymerisation. Known hyperbranched polymers have irregularly branched structures with high degrees of branching between 0.2 and 0.8. The degree of branching DB of an AB_2 hyperbranched polymer has been defined by the equation $DB=(1-f)$ in which f is the mole fraction of AB_2 monomer units in which only one of the two B groups has reacted with an A group.

Porphyrins occur widely in nature, and perform very important roles in various biological processes. The chemical structure of porphyrin is shown in Formula 1.

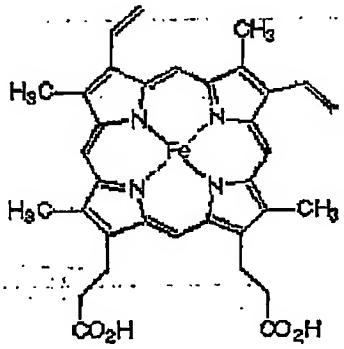


(1)

The basic structure of a porphyrin consists of four pyrrole units linked by four methine bridges. A feature of porphyrins is their ability to be metalated and demetalated. A number of metals (e.g. Fe, Zn, Cu, Ni) can be inserted into the porphyrin cavity by using various metal salts. Removal of the metal (demetalation) can be achieved, for example, by acid treatment.

Porphyrin can be synthesised by a variety of methods, for example, by tetramerisation of monopyrroles, by condensation of dipyrrolic intermediates, or by cyclization of open-chain tetrapyrroles.

Haem (the iron(II) protoporphyrin-IX complex) is the prosthetic group in haemoglobins and myoglobins, which are molecules responsible for dioxygen transport and storage in living tissues. Its chemical structure is shown in Formula 2.



(2)

Haemoglobin contains four protein subunits, each possessing a porphyrin moiety in their "active site". An iron (II) atom is located in the centre of each porphyrin moiety and it is this that reversibly binds dioxygen. An important role of the protein backbone is to protect and isolate the porphyrin active site within a hydrophobic environment.

Haem can also be found in the enzyme peroxidase, which catalyzes the oxidation of substrates with hydrogen peroxide. The related enzyme catalase, also containing haem, catalyzes the breakdown of hydrogen peroxide to water and oxygen. Other haem-containing proteins include the cytochromes, which serve as one-electron carriers in the electron transport chain.

Reduction of one of the pyrrole units on the porphyrin ring leads to a class of porphyrin derivatives called chlorins. Chlorophylls, found abundantly in green plants, belong to this category, and play an important role in the process of photosynthesis.

Recently, attempts have been made to prepare covalently linked multiporphyrin arrays, and to use such systems in artificial photosynthesis. The incorporation of porphyrin moieties into the framework of a dendrimer has been described by Jiang and Aida, J. Macromol. Sci, Pure Appl. Chem.,1997,A34,2047 and by Weyermann and Diederich J. Chem, Soc., Perkins Trans. 1, 2000, 4231 – 4233. The main drawback of dendrimers is that they have to be constructed by a multi-step synthesis, which is both lengthy and costly.

Hyperbranched aliphatic polyether polymers containing multiple porphyrin moieties have been described by Hecht et al in Chem. Commun., 2000, 313-314. These polymers have been suggested for use in photophysical and electrochemical studies, and for the

construction of optoelectronic devices, but they are of limited use in biological systems because of their bio-incompatibility and relative insolubility in biological media.

According to the present invention there is provided a water-soluble hyperbranched polymer comprising a porphyrin moiety.

In a first aspect, the present invention provides a water-soluble hyperbranched polymer comprising a porphyrin moiety and one or more hyperbranched polymer chains covalently bound thereto.

Preferably the porphyrin moiety is a focal core of the polymer and is surrounded by up to four hyperbranched polymer chains covalently bound thereto.

In a second aspect the invention provides a process for the production of a water-soluble hyperbranched polymer comprising one or more porphyrin moieties, which process comprises subjecting an AB_2 monomer to a polymerisation reaction in the presence of a functionalised porphyrin or porphyrin derivative as a polymerisation initiator core.

In a further aspect, the invention provides a water-soluble hyperbranched polymer comprising a porphyrin moiety having an Fe (II) atom inserted therein.

In a yet further aspect the invention provides a synthetic blood product which comprises an aqueous solution of a water-soluble hyperbranched polymer comprising a porphyrin moiety having an Fe (II) atom inserted therein capable of reversibly binding oxygen thereto.

Hyperbranched polymers of the present invention preferably have the structure:

$$P(HB)_n \qquad (3)$$

where P is a porphyrin moiety as hereinafter defined, HB is a hyperbranched polymer chain and n is an integer of from 1 to 4.

By "water-soluble" in this specification is meant that the hyperbranched polymers are soluble in water at least to the extent of 1g/l, more preferably at least 50 g/l, most preferably at least 100 g/l. The hyperbranched polymers of the present invention may be water-soluble, for example, due to the presence of solubilising substituents in the polymer chains. Neutral hydroxyl groups are particularly effective as solubilising substituents, although groups such as amine, acid, quaternary ammonium and other similar groups can also be used. The water-soluble polymer chains can, of course, comprise several different solubilising substituents. The solubilising substituents can be derived from the monomeric component(s) of the hyperbranched polymer chains, or can be introduced by substitution reactions.

The hyperbranched polymer chains can be, for example, polyethers, polyesters, and polyamides. Polyglycerols and other hydroxyl-substituted polyethers, are particularly preferred.

Where the hyperbranched polymer is a polyether, it can be derived from the polymerisation of AB_2 monomers such as, for example, 2-(bromomethyl)-2-methylpropane-1,3-diol (or derivatives thereof).

Where the hyperbranched polymer is a polyester, it can be derived from the polymerisation of AB_2 monomers such as, for example, 2,2-

bis(hydroxymethyl)butanoic acid, and 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoic acid.

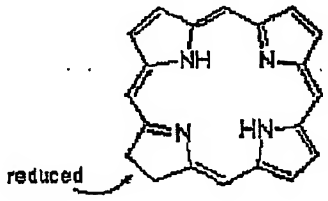
Latent AB_2 monomers, wherein the monomer polymerises by ring opening polymerisation are especially preferred. Preferred examples of latent AB_2 monomers include glycidol (2,3-epoxy-1-propanol), 2,3-epoxy-1-butanol, 2,3-epoxy-1-pentanol, and 4-(2-hydroxyethyl)-ε-caprolactone (and simple derivatives thereof).

The polymerisation reaction can be carried out, for example, under reflux in an organic solvent, preferably at a temperature of from 40 to 180 °C.

The hyperbranched polymer chains covalently linked to the porphyrin moiety preferably are of a size, shape and number sufficient to provide a hydrophobic region around the porphyrin moiety, to protect and isolate the porphyrin moiety. This is particularly important where the porphyrin moiety comprises an inserted ferrous ion, in order to reduce the rate of oxidation (and hence inactivation) of the ferrous ion. Preferably there are four hyperbranched polymer chains covalently linked to the porphyrin moiety for maximum protection. The hyperbranched polymers of the invention preferably have a molecular weight within the range of from 1000 to 10,000, more preferably from 4000 to 7000, most preferably from 5000 to 6000. The hyperbranched polymers preferably have a polydispersity of from 1.1 to 3.0.

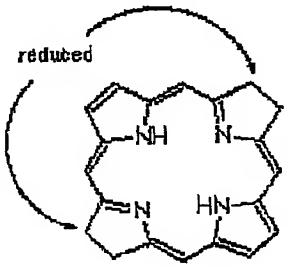
By "a porphyrin moiety" in this specification is meant a moiety having a basic structure of four linked pyrrole units, and derivatives thereof, including porphyrin (Formula I); alkyl substituted porphyrins, for example, C_{1-6} tetra (hydroxylalkyl) substituted porphyrins; aryl substituted porphyrins, for example, tetraphenol porphyrin; metalated

derivatives of porphyrin, for example, iron(II) protoporphyrin-IX complexes (Formula 2); reduction products of porphyrin, for example, chlorin (Formula 3);



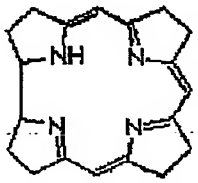
(3)

reduction products of chlorins in which the reduced pyrrole units are diagonally opposite to each other, for example, bacteriochlorins (Formula 4);



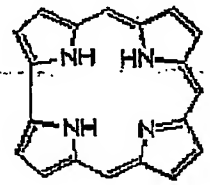
(4)

and porphyrin-like moieties such as corrin (Formula 5);



(5)

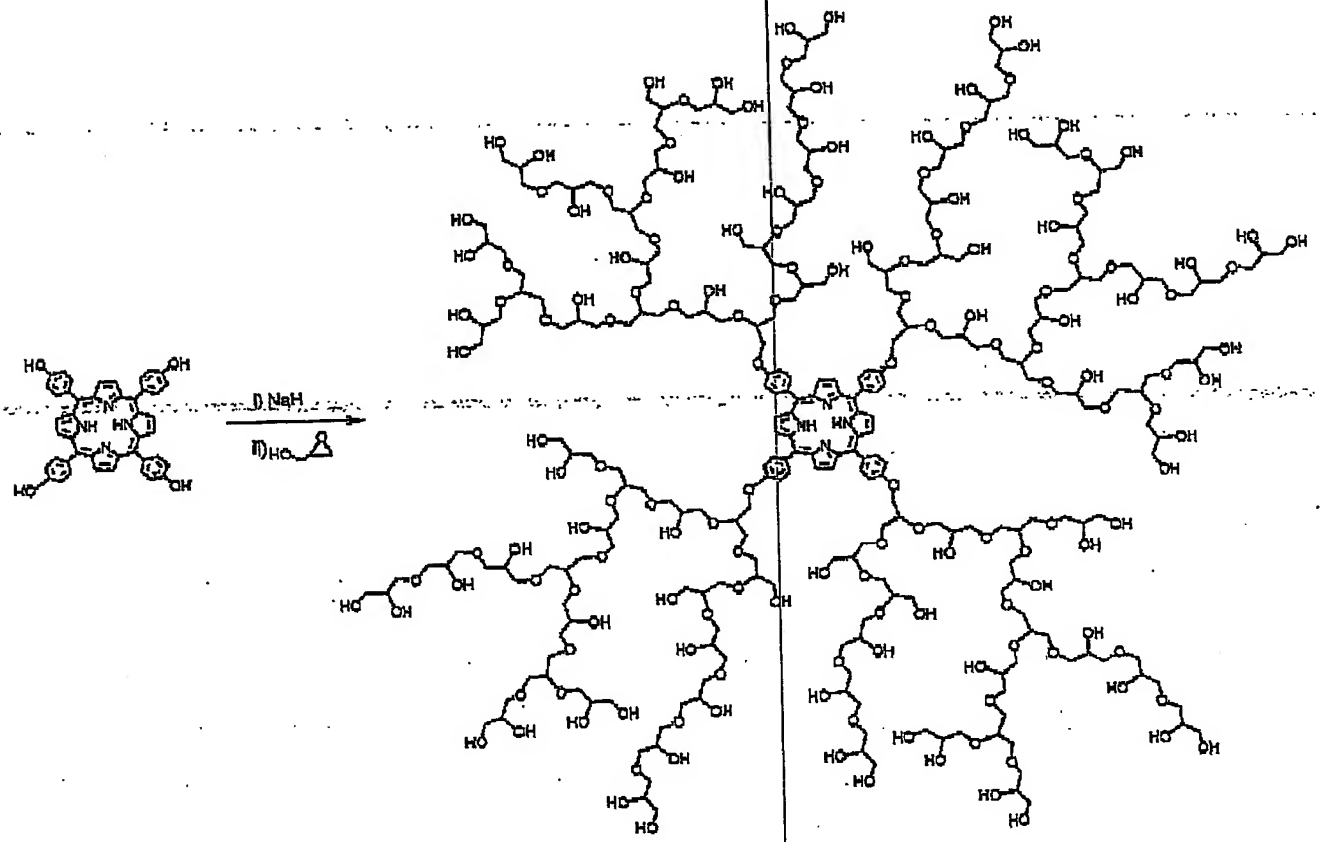
and corrole (Formula 6).



(6)

The functionalised porphyrin or porphyrin derivative is one that is capable of initiating the polymerisation of an AB_2 monomer and of forming a covalent bond with the growing hyperbranched polymer. The functional groups can be any of those capable of reacting with an AB_2 monomer, including hydroxyalkyl groups, hydroxyaryl groups, acid groups, amine groups, epoxy and ester groups. Functional groups can be introduced at any convenient location on the ring of the porphyrin or porphyrin derivative, provided that they do not inactivate the porphyrin. Thus substitutions can be made in the pyrrole rings or in the methine bridging groups as appropriate. Up to eight functional groups capable of initiating the polymerisation of an AB_2 monomer and of forming a covalent bond with the growing hyperbranched polymer can be introduced although four are often sufficient. Preferred functionalised porphyrin and porphyrin derivatives include especially 5, 10 , 15 , 20 – substituted porphyrins, particularly 5, 10, 15, 20 – hydroxyaryl substituted porphyrins, for example, 5, 10, 15, 20 – tetraphenol porphyrin and 5, 10, 15, 20 – tetra(dihydroxyphenyl) porphyrin. Such compounds can be activated to become polymerisation initiators, for example, by reaction with a deprotonating agent such as, for example, sodium hydride.

By "a focal core" in this specification is meant a region from which the hyperbranched polymer chains appear to radiate. In a preferred process according to the invention, a functionalised porphyrin or derivative is reacted with an AB_2 monomer under polymerisation conditions such that the AB_2 monomer polymerises to form hyperbranched polymer chains radiating from the porphyrin moiety which occupies the centre or core of the polymer molecule. An example of a polymerisation reaction according to the invention, using glycidol as the latent AB_2 monomer and 5, 10, 15, 20 – tetraphenol porphyrin as the reaction initiator, is illustrated in reaction scheme I:



(I)

Especially useful polymers in accordance with the invention are those in which the porphyrin moiety is metalated, preferably with an Fe(II) ion. The metalation can be carried out using iron salts, for example, ferrous chloride or ferrous bromide. Preferred embodiments of such polymers, in the presence of an axial ligand, are capable of mimicking the oxygen binding properties of blood and can be used as haemoglobin replacements. A wide range of axial ligands can be used in this aspect of the invention including nitrogen donor ligands such as, for example, pyridines, imidazoles and histidines. A particularly preferred donor ligand is 1, 2 – dimethylimidazole, The axial ligand is preferably present during the metalation step in order to stabilise the porphyrin complex.

Solutions of such polymers in a physiologically compatible fluid can also be used as synthetic blood products and as blood substitutes, for example, in emergency treatments.

Other embodiments of hyperbranched polymers according to the invention can be used as catalysts and in photodynamic therapy.

The invention is illustrated by the following non-limitative Example:

EXAMPLE

Synthesis of porphyrin centred hyperbranched polyglycerol .

The reaction was carried out in accordance with reaction scheme 1. Polymerization was carried out in a round bottomed flask equipped with a magnetic stirrer bar and a reflux condenser (under a nitrogen atmosphere). Tetraphenol porphyrin (1) (1.0g, 1.48mmol) in tetrahydrofuran (15 ml) was deprotonated using sodium hydride (0.071g, 2.9mmol). A 15 mL solution of glycidol (5.46g, 80mmol) in

ethylene glycol dimethyl ether was then added at 65° C over 12 hours *via* a syringe pump. The THF was then removed under vacuum to leave a red paste at the bottom of the flask. The excess ethylene glycol dimethyl ether was then decanted off and the crude product dissolved in methanol and twice precipitated into acetone. After drying (15h, 80° C, under vacuum), porphyrin centred polyglycerol was obtained as a red highly viscous paste in 45% yield (no trace of monomer or porphyrin could be detected by GPC). δ_H(250MHz, D_2O): 7.38(d(b), Ph-*H*), 6.95(d(b), Ph-*H*), 6.62(s(b), β-*H*) 4.91(s, O*H*), 4.05-3.15(m, C*H* and CH_2). GPC (water; pH 7.4), Mn 6507, Mw 7960 (DP ~ 80). UV:λ_{max} 418nm. The solubility of the porphyrin centred hyperbranched polyglcerol in water was 100mg/ml measured at 24°C.

Synthesis of Fe(II) – 1, 2 – dimethylimidazole porphyrin centred hyperbranched polyglycerol.

The porphyrin centred hyperbranched polyglycerol can be metalated by refluxing the polymer with $FeBr_2$ and pyridine in methanol as a solvent. The resultant Fe(III) porphyrin centred hyperbranched polyglycerol is reduced by reaction with an sodium dithionite $Na_2S_2O_4$. In order to produce an oxygen binding polymer, the reduction is preferably carried out in the presence of the axial ligand 1, 2 –dimethylimidazole.

Measurement of reversible O_2 binding

The ability of the Fe(II) centred hyperbranched polymers (HBP) of the invention to bind oxygen can be demonstrated as follows:

The experiments are carried out using water (degassed) as solvent, in a quartz UV cuvette (1cm path length) fitted with a suba seal. Oxygen is then bubbled through a solution of Fe(II) centred HBP containing a four fold excess of the axial ligand 1, 2 -dimethylimidazole for 1 minute. A UV spectrum of the solution is then measured and a clear and characteristic shift in the Soret band of the porphyrin is observed (i.e. from Fe(II) to the Fe(II)/O_2 complex). The position of the Soret band returns to the peak corresponding to Fe(II) after bubbling nitrogen through the same solution for 5 minutes. This procedure (O_2 followed by N_2) is then repeated 4 times, clearly demonstrating that the Fe(II) HBP is capable of reversibly binding O_2. With successive cycles (O_2 followed by N_2) irreversible oxidation begins to occur, as characterised by a peak corresponding to Fe(III) which begins to appear in the spectrum.

The reader's attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Each feature disclosed in this specification (including any accompanying claims, abstract and drawings), may be replaced by alternative features serving the same, equivalent, or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated

otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features.

The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

CLAIMS

1. A water-soluble hyperbranched polymer comprising a porphyrin moiety and one or more hyperbranched polymer chains covalently bound thereto.

2. A polymer according to claim 1, wherein the porphyrin moiety is a focal core of the polymer and is surrounded by up to four hyperbranched polymer chains covalently bound thereto.

3. A polymer according to claim 1 or 2, having the structure of Formula 3:

$$P(HB)_n \qquad (3)$$

where P is a porphyrin moiety as hereinafter defined, HB is a hyperbranched polymer chain and n is an integer of from 1 to 4.

4. A polymer according to any one of the preceding claims, wherein the hyperbranched polymer is soluble in water at least to the extent of 1 g/l.

5. A polymer according to any one of the preceding claims , wherein the hyperbranched polymer is provided with solubilising substituents in the polymer chains.

6. A polymer according to claim 5, wherein the solubilising substituent is selected from hydroxyl groups, amine groups, acid groups, and quaternary ammonium groups.

7. A polymer according to claim 5 or 6, wherein the solubilising substituents are derived from the monomeric component(s) of the hyperbranched polymer chains.

8. A polymer according to any one of the preceding claims, wherein the hyperbranched polymer chains are polyethers, polyesters or polyamides.

9. A polymer according to claim 8, wherein the hyperbranched polymer chains are polyglycerols or other hydroxyl-substituted polyethers.

10. A polymer according to any one of the preceding claims, wherein the hyperbranched polymer chains covalently linked to the porphyrin moiety are of a size, shape and number sufficient to provide a hydrophobic region around the porphyrin moiety, to protect and isolate the porphyrin moiety.

11. A polymer according to claim 10, wherein there are four hyperbranched polymer chains covalently linked to the porphyrin moiety.

12. A polymer according to any one of the preceding claims, wherein the hyperbranched polymer has a molecular weight within the range of from 2000 to 24000.

13. A polymer according to any one of the preceding claims, wherein the hyperbranched polymer has a polydispersity of from 1.1 to 3.0.

14. A polymer according to any one of the preceding claims, wherein the porphyrin moiety is selected from porphyrin; alkyl substituted porphyrins; aryl substituted porphyrins; metalated derivatives of porphyrin; chlorins; bacteriochlorins; corrins; and corroles.

15. A polymer according to claim 18, wherein the porphyrin moiety is an iron(II) protoporphyrin-IX complex.

16. A polymer according to any one of the preceding claims substantially as described in the Example.

17. A hyperbranched polymer according to any one of the preceding claims substantially as hereinbefore described.

18. A process for the production of a water-soluble hyperbranched polymer comprising one or more porphyrin moieties, which process comprises subjecting an AB_2 monomer to a polymerisation reaction in the presence of a functionalised porphyrin or porphyrin derivative as a polymerisation initiator core.

19. A process according to claim 18, wherein the hyperbranched polymer is a polyether and the AB_2 monomer is selected from, 2-(bromomethyl)-2-methylpropane-1,3-diol or simple derivatives thereof.

20. A process according to claim 18, wherein the hyperbranched polymer is a polyester and the AB_2 monomer is selected from 4-(2-hydroxyethyl)-ε-caprolactone, 2,2-bis(hydroxymethyl)butanoic acid and 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoic acid.

21. A process according to claim 18, wherein the polymer is derived from a latent AB_2 monomer, wherein the monomer polymerises by ring opening polymerisation.

22. A process according to claim 21, wherein the latent AB_2 monomer is selected from glycidol (2,3-epoxy-l-propanol), 2,3-epoxy-l-butanol, 2,3-epoxy-l-pentanol, and 4-(2-hydroxyethyl)-ε-caprolactone.

23. A process according to any one of claims 18 to 22, wherein the functionalised porphyrin or porphyrin derivative comprises functional groups selected from hydroxyalkyl groups, hydroxyaryl groups, acid groups, amine groups, epoxy groups and ester groups.

24. A process according to any one of claims 18 to 23, wherein the functionalised porphyrin or porphyrin derivative is a 5, 10, 15, 20 – substituted porphyrin.

25. A process according to claim 24, wherein the functionalised porphyrin or porphyrin derivative is 5, 10, 15, 20 – tetraphenol porphyrin.

26. A process according to any one of claims 18 to 25, wherein the functionalised porphyrin or porphyrin derivative is activated by reaction with a deprotonating agent.

27. A process according to any one of claims 18 to 26, wherein a functionalised porphyrin or derivative is reacted with an AB_2 monomer under polymerisation conditions such that the AB_2 monomer polymerises to form hyperbranched polymer chains

radiating from the porphyrin molety which occupies the centre or core of the growing polymer molecule.

28. A process according to any of claims 18 to 27, which is carried out under reflux in an organic solvent, at a temperature of from 40 to 180 °C.

29. A process according to any of claims 18 to 28 substantially as described in the Example.

30. A process according for the production of a hyperbranched polymer substantially as hereinbefore described.

31. A water-soluble hyperbranched polymer comprising a porphyrin molety having a metal ion inserted therein.

32. A water-soluble hyperbranched polymer according to claim 31, wherein the metal ion is an Fe (II) ion.

33. A water-soluble hyperbranched polymer according to claim 31 or 32, wherein the polymer is a polymer as claimed in any one of claims 1 to 17.

34. A water-soluble hyperbranched polymer according to any one of claims 31 to 33, wherein the metal ion is associated with an axial ligand.

35. A water-soluble hyperbranched polymer according to claim 34, wherein the axial ligand is a nitrogen donor ligand.

36. A water-soluble hyperbranched polymer according to claim 34 or 35, wherein the axial ligand is a pyridine, imidazole or histidine.

37. A water-soluble hyperbranched polymer according to any one of claims 34 to 36, wherein the axial ligand is 1, 2 – dimethylimidazole.

38. A water-soluble hyperbranched polymer according to any one of claims 34 to 37, which is capable of reversibly binding oxygen thereto.

39. A water-soluble hyperbranched polymer according to any one of claims 31 to 38, substantially as described in the Example.

40. A water-soluble hyperbranched polymer capable of reversibly binding oxygen thereto substantially as hereinbefore described.

41. A process for the production of a polymer according to any one of claims 31 to 40, wherein a polymer according to any one of claims 1 to 17 is reacted with a metal salt.

42. A process according to claim 41, wherein the metal salt is a ferrous salt.

43. A process according to claim 41 or 42 wherein the reaction takes place in the presence of an axial ligand.

44. A process according to claim 43, wherein the axial ligand is 1, 2 – dimethylimidazole.

45. A process according to any one of claims 39 to 44, substantially as described in the Example.

46. A process according to any one of claims 39 to 45, substantially as hereinbefore described.

47. A synthetic blood product or blood substitute, which comprises an aqueous solution of a water-soluble hyperbranched polymer comprising a porphyrin moiety having an Fe (II) atom inserted therein capable of reversibly binding oxygen thereto.

48. A synthetic blood product according to claim 47, wherein the hyperbranched polymer is a polymer as claimed in any one of claims 31 to 40.

49. Use of a polymer as claimed in any one of claims 32 to 40 as a replacement for haemoglobin.

50. Use of a polymer according to any one of claims 1 to 17 as a catalyst or in photodynamic therapy.

ABSTRACT

A water-soluble hyperbranched polymer comprising a porphyrin moiety and one or more hyperbranched polymer chains covalently bound thereto. The polymers when metalated with an Fe(II) ion are capable of mimicking the oxygen binding properties of blood. The polymers may be used as haemoglobin replacements and in synthetic blood products and as blood substitutes.